Adaptation of Saccharomyces cerevisiae to saline stress through laboratory evolution

Dhar, R ; Sägesser, R ; Weikert, C ; Yuan, J ; Wagner, A

Abstract: Most laboratory evolution studies that characterize evolutionary adaptation genomically focus on genetically simple traits that can be altered by one or few mutations. Such traits are important, but they are few compared with complex, polygenic traits influenced by many genes. We know much less about complex traits, and about the changes that occur in the genome and in gene expression during their evolutionary adaptation. Salt stress tolerance is such a trait. It is especially attractive for evolutionary studies, because the physiological response to salt stress is well-characterized on the molecular and transcriptome level. This provides a unique opportunity to compare evolutionary adaptation and physiological adaptation to salt stress. The yeast Saccharomyces cerevisiae is a good model system to study salt stress tolerance, because it contains several highly conserved pathways that mediate the salt stress response. We evolved three replicate lines of yeast under continuous salt (NaCl) stress for 300 generations. All three lines evolved faster growth rate in high salt conditions than their ancestor. In these lines, we studied gene expression changes through microarray analysis and genetic changes through next generation population sequencing. We found two principal kinds of gene expression changes, changes in basal expression (82 genes) and changes in regulation (62 genes). The genes that change their expression involve several well-known physiological stress-response genes, including CTT1, MSN4 and HLR1. Next generation sequencing revealed only one high-frequency single-nucleotide change, in the gene MOT2, that caused increased fitness when introduced into the ancestral strain. Analysis of DNA content per cell revealed ploidy increases in all the three lines. Our observations suggest that evolutionary adaptation of yeast to salt stress is associated with genome size increase and modest expression changes in several genes.

DOI: https://doi.org/10.1111/j.1420-9101.2011.02249.x

Title: Adaptation of *Saccharomyces cerevisiae* to saline stress through laboratory evolution

Riddhiman Dhar[1,3], Rudolf Sägesser[1,3], Christian Weikert[1,3], Ju Yuan[1,3] and Andreas Wagner[1,2,3,4]

[1]Dept. of Evolutionary Biology and Environmental Studies, University of Zurich

[2] The Santa Fe Institute

[3] The Swiss Institute of Bioinformatics

[4] Author for correspondence
Address:
University of Zurich
Dept. of Biochemistry, Bldg. Y27
Winterthurerstrasse 190
CH-8057 Zurich
Switzerland
Email: aw@bioc.uzh.ch
Phone: +41-44-635-6141
Fax: +41-44-635-6144

Formatiert: Französisch (Schweiz)

Abstract

Most laboratory evolution studies that characterize evolutionary adaptation genomically focus on genetically simple traits that can be altered by one or few mutations. Such traits are important, but they are few compared to complex, polygenic traits influenced by many genes. We know much less about complex traits, and about the changes that occur in the genome and in gene expression during their evolutionary adaptation. Salt stress tolerance is such a trait. It is especially attractive for evolutionary studies, because the physiological response to salt stress is well-characterized on the molecular and transcriptome level. This provides a unique opportunity to compare evolutionary adaptation and physiological adaptation to salt stress. The yeast *Saccharomyces*

cerevisiae is a good model system to study salt stress tolerance, because it contains several highly conserved pathways that mediate the salt stress response. We evolved three replicate lines of yeast under continuous salt (NaCl) stress for 300 generations. All three lines evolved faster growth rate in high salt conditions than their ancestor. In these lines, we studied gene expression changes through microarray analysis, and genetic changes through next generation population sequencing. We found two principal kinds of gene expression changes, changes in basal expression (82 genes), and changes in regulation (62 genes). The genes that change their expression involve several well-known physiological stress response genes, including *CTT1, MSN4, HLR1*. Next generation sequencing revealed only one high frequency single nucleotide change, in the gene *MOT2*, that caused increased fitness when introduced into the ancestral strain. Analysis of DNA content per cell revealed ploidy increases in all the three lines. Our observations suggest that *evolutionary* adaptation of yeast to salt stress is associated with genome size increase and modest expression changes in several genes.

Introduction

The ability to characterize the changes that occur during evolutionary adaptation on a genome-wide scale has been a boon for the field of laboratory evolution. Most published studies focus on traits with a simple basis, where changes of major effects in one or few changes can alter a trait during laboratory evolution experiments (Ferea *et al.*, 1999; Blanc & Adams, 2003; Velicer *et al.*, 2006; Stanek *et al.*, 2009). Such traits are important, but they are in the minority. The vast majority of traits have a complex, polygenic basis (refs. Benfey, Genomics textbook). We know much less about how genomic change and change in gene expression occurs in such polygenic traits. Our study is a step towards answering this question. We here focus on a prototypical polygenic trait, an organism's response to high concentrations of salt in its environment. The physiological response of an organism to such salt stress is well-studied on the molecular and the transcriptome levels (Posas *et al.*, 2000; Rep *et al.*, 2000; Causton *et al.*, 2001; Yale & Bohnert, 2001). This fact provides another important motivation to study the salt stress response in an evolutionary context. It allows us to ask whether evolutionary adaptation to salt stress is similar to physiological adaptation, using a genome-scale approach that relies on transcriptome changes in response to salt stress.

Environmental fluctuations and stressors constantly challenge organisms in the wild. Organisms thus use cellular mechanisms to adapt to and to survive environmental fluctuations. Hyperosmotic stress is one prominent environmental

stressor, where a cell experiences higher solute concentration outside the cell than inside. This causes water loss from the cell, resulting in a higher intracellular concentration of ions and metabolites, and eventual arrest of cellular activity. Hyperosmotic stress is caused by high concentrations of sugar or salt, e.g., sodium chloride (NaCl). High salt stress is a special case of hyperosmotic stress and has similar effects on a cell as high concentration of sugars (Gasch *et al.*, 2000; Causton *et al.*, 2001). In addition, it causes hyperionic stress due to high extracellular concentrations of Na^+ and Cl^- ions, which are imported into the cell and can disrupt cellular ionic equilibrium. Tolerance to Na^+ stress thus needs additional ion transport and detoxification mechanisms along with those required for the hyperosmotic stress response (Serrano, 1996; Apse *et al.*, 1999; Gaxiola *et al.*, 1999; Maathuis & Amtmann, 1999; Serrano *et al.*, 1999; Zhu, 2001). The hyperosmotic stress response in yeast is mediated by the high osmolarity glycerol (HOG) pathway, which is a MAPK pathway (Brewster *et al.*,1993; Dihazi *et al.*, 2004, Saito & Tatebayashi, 2004; reviewed in Hohmann 2002).Two cell membrane-bound sensors Sho1p and Sln1p detect osmotic change, which results in activation of Hog pathway genes, which, in turn, leads to the activation of the downstream genes associated with salt tolerance and adaptation (Martinez-Pastor *et al.*, 1996; Schmitt & McEntee, 1996; Gorner *et al.*, 1998; Ostrander & Gorman, 1999; Rep *et al.*, 1999; Reiser *et al.*, 2000; Rep *et al.*, 2000). Whole transcriptome studies by Gasch *et al*, Posas *et al*, Yale and Bonhart, Causton *et al.*, and Rep *et al.* (Gasch *et al.*, 2000; Posas *et al.*, 2000; Rep *et al.*, 2000; Causton *et al.*, 2001; Yale & Bohnert, 2001) have identified hundreds of genes whose expression levels are affected by hyperosmotic stress. The genes induced during osmotic stress response include the genes involved in synthesis and regulation of the cellular osmolytes glycerol and trehalose. Another group of genes, specifically activated under saline stress, are associated with ion homeostasis. Some of the genes affected by osmotic stress show transient expression changes. whereas others show expression changes that are stable in time. In addition to genome wide approaches, many studies have characterized the roles of individual genes in the osmotic stress response of yeast (Haro *et* al., 1991; Garciadeblas *et al.,* 1993; Mai & Breeden, 1997; Ganster *et al.*, 1998; Mendizabal *et al.*,1998; Tsujimoto *et al.*, 2000; Betz *et al.*, 2002; Goossens *et al.*, 2002**;** Hirata *et al.*, 2003; Heath *et al.*, 2004;).

Yeast is a good model system for studying osmo-adaptation in eukaryotes, since other fungi and plants share many of the stress response pathways and proteins involved in osmo-adaptation in yeast. First, the mitogen-activated protein kinase (MAP kinase) cascade, central to the stress response in yeast, is a conserved eukaryotic signal transduction pathway present from fungi to plants (Pelech & Sanghera, 1992; Errede & Levin, 1993; Nishida & Gotoh, 1993; Neiman, 1993; Neiman *et al.*, 1993; Kosako *et* al., 1993; Tsuda *et al.*, 1993; Yashar *et* al., 1993; Cooper, 1994; Zamanillo *et al.*, 1994; Sanchez *et* al., 1994; Yan *et al.*, 1994; Galcheva-Gargova *et* al., 1994; Han *et al.*, 1994; Waskiewicz & Cooper, 1995; Hirt, 1997; Jonak *et al.*,1999; Kovtun *et* al., 2000; Kyriakis & Avruch, 2001). Stress signaling in plants is also carried out by MAPK pathways, which are activated by cold, drought, salt, heat, and oxidative stress (Jonak *et* al., 1996; Kovtun *et* al., 2000; Teige *et al.*, 2004). Second, yeast and plants have

highly similar genes required for stress tolerance (Mendoza *et al.*, 1994; Bressan *et al.*, 1998; Pardo *et al.*, 1998; Lee *et al.*, 1999; Sanders, 2000; Hasegawa & Bressan, 2000; Quintero *et al.*, 2002; Zhu, 2002). Third, yeast and plants have similar membrane ion transport and detoxification systems (Gaxiola *et al.*, 1999; Quinteroa *et al.*, 2000). For example, HAL family genes are important for ion homeostasis in yeast as well as in plants (Haro *et al.*, 1991; Gaxiola *at al.*, 1992; Ferrando *et al.*, 1995; Murguía *et al.*, 1996; Rios *et al.*, 1997; Mulet *et al.*, 1999; Espinosa-Ruiz *et al.*, 1999; Gisbert *et al.*, 2000; Yang *et al.*, 2001).

With one exception (Samani & Bell, 2010), all previous studies of salt stress adaptation in yeast focused on *physiological* adaptation. Such adaptation occurs on time scales up to a few hours. The mechanisms of longer term *evolutionary* adaptation to salt stress are not known. Such adaptations occur on time scales of hundred generations or more. One aim of this study is to compare a population's evolutionary response to salt stress with its physiological response on the transcriptome level. Does evolutionary adaptation mirror physiological adaptation? Does it affect largely the same genes as the physiological response?

A second aim is to investigate the genetic basis of *evolutionary* adaptation of yeast to high saline stress, as far as this is possible for a complex trait. Does the adaptation come about through accumulation of identifiable beneficial point mutations? Does it involve chromosomal rearrangements as observed in evolution of yeast in glucose-limited or phosphate-limited media? Or does it take place through genetic and epigenetic changes altering expression of genes that help cells adapt to high salt? These are some of the questions we ask.

Understanding of *evolutionary* principles of salt tolerance could also be important for biotechnological applications. Firstly, yeast cells experience high salt concentration in many industrial fermentation processes, and improvement in performance of yeast in such conditions would benefit the industry immensely (Attfield, 1997; Trainotti & Stambuk, 2001; Zheng *et al.*, 2011). Secondly, the principles of salt tolerance in yeast might be useful for engineering fungi and crop plants for salt tolerance, as both classes of organisms share many components of their stress response systems.

In this contribution, we exposed yeast to high salt concentrations for 300 generations in the laboratory in medium containing sodium chloride (NaCl). We compared the fitness and viability of the evolved lines with the starting yeast strain (ancestral strain), followed by characterization of gene expression changes and genetic changes such as mutations, copy number variations and chromosomal alterations.

Results

To test for evolutionary adaptation of yeast cells to osmotic stress, we carried out laboratory evolution experiments through 30 serial transfers in batch cultures, comprising approximately 300 cell generations (see Methods). This number of generations is sufficient to show evolutionary adaptations in yeast (Adams & Oeller, 1986; Adams *et al.*, 1992; Dunham *et al.*, 2002; Gresham *et al.*, 2008). For our experiments, we used haploid populations to avoid any potential masking of adaptively

significant alleles in the diploid stage (Zeyl *et al.*, 2003). We carried out three parallel replicate evolution lines in which yeast cells grew and divided in yeast peptone medium supplemented with galactose as the sole carbon source and with 0.5M NaCl (YPGN), which is a high salt medium that exposes cells to high osmolarity stress. We refer to these lines as S lines (S1, S2 and S3). As a control, we also carried out three replicates where the growth medium did not contain NaCl (lines C1,C2 and C3).

Evolutionary adaptation to NaCl

Before embarking on our experiments, we asked whether NaCl affects the fitness of our ancestral strain. Only in this case would we expect that NaCl exerts a selection pressure to which the strain can adapt. Fitness has two main components in our experiments. These are viability on the one hand, and growth or cell division rate on the other hand. We found that osmotic stress does not affect viability of the ancestral strain significantly (Figure 1a). Not surprisingly then, viability does not increase over the course of our experiment for lines evolved on NaCl (Figure 1b) and for lines evolved without NaCl (Figure 1c).

In contrast to viability, population growth rate is affected by salt stress, as shown in supplementary figure 1. The figure indicates that the ancestral strain grows significantly more slowly in medium containing NaCl. Thus, the fitness of the ancestral strain is lower in salt compared to that of the ancestral strain in medium without any salt. Having established that NaCl does affect the fitness of the ancestral strain through its growth rate, we asked if the growth rate and thus, relative fitness w (see Methods) of three salt evolved lines have increased after 300 generations of evolution (Figure 2a). Fitness increased significantly relative to the ancestral strain, such that the final evolved lines had a relative population mean fitness between w=1.11 and w=1.17, which corresponds to a decrease in the average cell doubling time between 8.2% and 12.3% relative to the ancestral strain (see Methods).

Since our high salt medium is a complex medium, it is likely that part of the evolutionary response we observe also reflects adaptation to medium components different from NaCl. To ask whether this is the case, we also measured the relative fitness of the three S lines in the control medium (without NaCl). Not surprisingly, the fitness in this medium had also increased (Figure 2b) relative to the ancestral strain, which suggests that at least part of the evolutionary adaptation we see is not specific to NaCl as a stressor. However, two lines of evidence show that a substantial fraction of the fitness increase is specific to NaCl. The first is that the fitness increase, when measured in the absence of NaCl, appears much lower than when measured in the presence of NaCl (Figures 2a and 2b)(t-test, $p<0.0001$ for S1, S2 and S3). The second line of evidence is provided by our three control lines that had evolved without the addition of NaCl to the medium. Figure 2a shows that in every single line the fitness increase, when measured in medium with NaCl, is consistently and significantly lower than for the lines that had evolved in NaCl (t-test, $p<0.0001$ for comparisons between S1 & C1, S2 & C2 and S3 & C3). Specifically, the increase in relative fitness w was at least 54 percent higher in the lines evolved in NaCl compared to the lines evolved without NaCl. In sum, a significant and substantial part of the evolutionary adaptation

we observe is due to the selection pressure provided by NaCl.

Gene Expression analysis of the evolved lines

To compare the expression levels of genes in the evolved lines and in the ancestral strain, we performed whole gene transcriptome analysis using yeast microarrays (Affymetrix). Because previous experiments had shown that many yeast genes change expression in response to salt and to other stressors (Gasch *et al.*, 2000; Posas *et al.*, 2000; Rep *et al.*, 2000; Causton *et al.*, 2001; Yale & Bohnert, 2001), we expected that this would also hold for our lines. Thus, we first asked which genes respond to salt in a similar manner in the ancestral strain and in all evolved lines. We will refer to such genes for brevity as *shared* genes. For all the analyses presented hereon we used all the replicates (see methods).

In all, we observed 581 shared genes that were induced in salt and 580 shared genes that were repressed in salt with 2-fold expression change ($|\log_2(\text{fold change})| \geq 1$, t-test, p-value<0.05, False Discovery Rate <10%). Figure 3a shows a "volcano plot" ($-\log_{10}$(p-value) vs $\log_2$(fold change)) for all the yeast genes in response to NaCl in the S lines and the ancestral strain. Figure 3b shows the extent of expression change in the evolved lines (vertical axis) and in the ancestral strain (horizontal axis) for all genes with similar response to NaCl in the evolved lines and the ancestral strain ($|\log_2(\text{fold change})| \geq 1$, t-test, p-value<0.05, False Discovery Rate <10%). The genes we labeled by name in the figure include some known stress response genes (e.g., *GPD1, SIP18*), some genes whose expression changed substantially (e.g., *FMP48, NOG1*), and genes in both categories (e.g., *GRE1*). Many of these shared genes are directly associated with saline stress, hyperosmotic stress, or the general stress response, and were shown to be affected by hyper-osmolarity and salt stress in previous studies of the *physiological* and osmotic stress response (Gasch *et al.*, 2000; Posas *et al.*, 2000; Rep *et al.*, 2000; Causton *et al.*, 2001; Yale & Bohnert, 2001). Among the 581 shared induced genes, 192 genes were shown to be affected by stress in previous studies; among the 580 shared repressed genes, 56 were affected by stress in previous studies.

Next, we classified shared genes using the Comprehensive Yeast Genome Database (CYGD) classification from the Munich Information Center for Protein Sequences (MIPS) (Güldener *et al.*, 2005). A detailed analysis is given in the supplementary material (supplementary figure 2 and supplementary text). Here, we only discuss two gene classes in more detail. First, genes associated with 'cell rescue, defense and virulence' contain many general stress response genes, as well as genes that respond specifically to saline stress. One would expect that such genes are induced in response to salt stress, and our data show that induced genes in this category are overrepresented, and repressed genes in this category are underrepresented (supplementary figure 2 and supplementary text). This group of genes was also found to be significantly overrepresented among genes whose deletion reduced the growth rate of yeast in salt (Warringer *et al.*, 2003). A second class of genes are genes involved in protein synthesis. The *physiological* response to stress can cause repression of protein synthesis (Gasch *et al.*, 2000). In support of this observation, our data show that significantly fewer genes associated with protein

synthesis are induced, and significantly more genes than expected are repressed (supplementary figure 2 and supplementary text). Again, this group of genes was significantly overrepresented among genes whose deletion increased salt-resistance (Warringer *et al.*, 2003).

Differentially Expressed Genes

While an analysis of genes regulated *similarly* in ancestral and evolved strains is instructive, we were more interested in genes that are expressed *differently*. These are genes whose expression adapted evolutionarily to salt stress. They can be subdivided into two main categories. The first comprises genes whose regulation has changed in the evolved lines. Figure 4a and b show two hypothetical examples of genes in this category. The second category comprises genes whose basal level of expression changed in the evolved lines, even in the absence of salt stress (Figure 4c and d). This second category of genes may be especially important, because our selection conditions imposed continuous salt stress. It is thus conceivable that cells whose expression is ancestrally regulated in response to salt stress simply increase or decrease their basal expression to the level of regulated expression in the ancestral strain in the absence of salt. We note that these two categories have multiple subcategories, and there can be genes where both the basal expression and regulation can change. Supplementary figure 3 shows an overview of all possibilities.

We distinguished genes in the main categories by calculating two different Z-scores for each gene, a *Z-score for change in basal expression (Z_b)* and a *Z-score for change in regulation (Z_r)* (see Methods) (Mukhopadhyay *et al.*, 2006). In this analysis, we considered genes with |Z-score|≥1.5 as differentially expressed.

Genes whose regulation changes

In all, there are 62 genes whose regulation changes during our experiment (Figure 5a). As we mentioned above, multiple types of such change are possible. Firstly, a gene's induction in salt can increase. This will occur if the gene is induced more strongly in the evolved lines than the ancestral strain (supplementary figure 3b). Secondly, a gene's induction in salt can become reduced (supplementary figure 3c). Thirdly, a gene's repression in salt can be increased, i.e., the gene becomes repressed to a greater extent in the evolved lines (supplementary figure 3d). Fourth, a gene's repression in salt can be reduced (supplementary figure 3e). Finally, a gene that was not regulated in the ancestral strain can become regulated in response to salt (supplementary figure 3f and g). Figure 5a plots the extent to which genes changed in their regulation against their Z_r scores. Some of the genes with significant changes in the regulation are labeled. Figure 6a and b show the five genes whose induction or repression changed most significantly (based on Z_r). We will discuss some of them further below.

There are only four genes in total which showed *increased induction* in the evolved lines. Among these four genes, two of them (*PUT4* and *PCL5*) also showed decrease in basal expression in the evolved lines. Four further genes showed *new induction* in the evolved lines. One of them, *CUP1-1* (Figure 5a), has previously been

observed to be up-regulated in response to osmotic stress (Yale & Bohnert, 2001). Five genes showed *reduced induction* in our experiment. One of the genes, *HSP30*, encodes a stress-responsive protein that negatively regulates the H(+)-ATPase Pma1p. It is induced by heat shock, treatment with organic acid and ethanol, and glucose starvation (Panaretou & Piper, 1992; Piper *et al.*, 1994; Piper *et al.*, 1997).

In contrast to the few genes which showed increased induction, a total of 37 genes showed *increased repression*. Out of these 37 genes, one gene (*HLR1*) is directly involved in the stress response. This gene encodes a protein involved in regulation of cell wall composition, as well as in the osmotic stress response (Alonso-Monge *et al.*, 2001). 12 genes showed *new repression* in our experiment. Among these 12 genes, the genes *GCV2* and *GCV1* were previously found to be up-regulated in osmotic stress (Yale & Bohnert, 2001). We did not find any gene with *reduced repression*.

We next classified genes whose regulation changed in any of these ways according to their functions, using the CYGD classification of yeast genes (Güldener *et al.*, 2005) (Figure 7), and compared their distribution among functional categories with the proportions of the yeast genome in each category. The distribution of the number of genes among different classes is non-random for genes whose repression changes (49 genes in total; Chi-square test, p-value<0.001, df=17), whereas the distribution is not significantly different from random for the genes whose induction changes (13 genes; p-value>0.1,df=17). We then asked whether genes whose regulation changes occur preferentially in specific functional categories. Interestingly, for genes with change in induction level, such genes are (marginally) enriched only in the functional class of cell rescue, defense and virulence (p=0.0444). Genes whose repression changes are enriched in the functional classes transcription (p=0.0006), protein synthesis (p=0.0008), and proteins with binding or catalytic function (p=0.0003). The classes protein fate (folding, modification, destination; p=0.0232), cellular transport (p=0.0048), cell cycle/DNA processing (p=0.0134) and cell rescue, defense and virulence (p=0.0230) contain fewer genes whose repression chages than expected by chance alone (all p-values based on an exact binomial test).

Genes with basal level expression change

Next we turn to genes that show a change in basal expression. There are 82 such genes. Thirty eight of them increased their basal expression, whereas 44 genes reduced their basal expression. Figure 5b plots the extent of change in basal expression ($\log_2$-transformed) of all the genes against their corresponding Z_b values. Some of the genes with significant changes in the basal expression level are labeled. Figure 6c and d lists the top five genes (based on Z_b) with increased and decreased basal expression, respectively. The distribution of genes among different functional classes (Figure 7) is significantly different from what would be expected by chance alone for the genes with increase in basal expression (p<0.05,df=17, Chi-square test), and also for the genes with a decrease in basal expression (p<0.001,df=17). For genes with an increase in basal expression, the class 'cell rescue and defense' (stress response) shows significantly more genes than expected (p=0.0059, exact binomial test), and the class

protein fate (p=0.0409) shows significantly fewer genes than expected by chance alone. For genes with a decrease in basal expression, the class 'cell type differentiation' (cell wall, sporulation, spore wall etc.) (p=0.0165) shows more genes than expected, and the classes 'transcription' (p=0.0125) as well as 'protein fate' (folding, modification, destination) (p=0.0014) contain fewer genes than expected.

Two well-established stress response genes showed an increase in basal expression in the evolved lines. One of them is *CTT1*, whose basal expression increased by ~1.6 fold compared to the ancestral strain. This gene was found to be induced in four previous genome-wide studies of *physiological* stress adaptation in yeast (Gasch *et al.*, 2000; Rep *et al.*, 2000; Causton *et al.*, 2001; Yale & Bonhert, 2001). The second known stress response genes is the transcription factor *MSN4*, whose expression was increased by approximately 1.5 fold. A more detailed analysis of individual genes can be found in the supplementary material (supplementary text).

Whole Genome (Re)Sequencing Analysis

Some laboratory evolution studies have identified few beneficial mutations of large fitness effects in evolving populations (Blanc & Adams, 2003; Velicer *et al.*, 2006; Stanek *et al.*, 2009). To find out if there are any such mutations, we subjected the ancestral strain and population samples from two of our evolved lines (S1 and S2) to deep sequencing at about ~10X coverage and the third line (S3) to ~50X coverage, using the Roche 454 Genome Sequencer. In our analysis of this sequence data, we aimed to identify only changes that may have swept to high frequency or fixation and 10X coverage is sufficient for that purpose. We chose candidate SNPs based on the criteria for the changed (derived) nucleotide described in supplementary methods and our approach is deliberately conservative. We identified 56 candidate SNPs and 7 deletions in total from all the three evolved (S) lines.

We sequenced all 56 candidate SNPs and 7 candidate deletions, but only one of them turned out to be a true change. This change was a SNP unique to line S2, and occurred at a frequency exceeding 50 percent, based both on next generation sequencing data and PCR sequencing data. This SNP is a non-synonymous G to A mutation (amino acid change: G230D) in the *MOT2* (YER068W) gene. The gene *MOT2* is a subunit of the CCR4-NOT complex which has roles in transcription regulation, mRNA degradation, and post-transcriptional modifications (Cade & Errede, 1994; Liu *et al.*, 1998; Badarinarayana *et al.*, 2000; Denis *et al.*, 2001; Panasenko *et al.*, 2006; Mersman *et al.*, 2009), with no known role in salt tolerance. To test whether the *MOT2* SNP alone provided any fitness benefit to yeast in salt, we replaced the wild-type variant of *MOT2* in the genome of the ancestral strain with the mutant (see Methods). Three replicate competition assays using FACS (see Methods) showed a relative population fitness w=1.043±0.008 for the mutant *MOT2* allele (Figure 8a) in medium with NaCl, compared to w=0.999±0.014 for the wildtype *MOT2* allele. However, the fitness increase of the mutant *MOT2* could only explain 25.3 percent of the total amount of fitness increase in line S2, whose relative fitness was w=1.17, indicating that there are other factors that contribute to the fitness increase of the line S2. In addition, we note that the *MOT2* mutation also conferred a substantial fitness increase in the

absence of NaCl (Figure 8b), indicating that this mutation is not specifically adaptive to salt stress.

Duplication and PFGE

We next turned to copy number variations as sources of evolutionary adaptation. Since large-scale chromosomal rearrangements and aneuploidies are frequent in yeast laboratory evolution (Adams *et al.*, 1992; Dunham *et al.*, 2002; Rancati *et al.*, 2008), and are also observed in yeast gene knock-out strains (Hughes *et al.*, 2000), we first wanted to know whether such rearrangements were numerous in our evolved lines. To this end, we first performed pulsed field gel electrophoresis (PFGE) analysis of whole chromosomes for two clones of the ancestral strain, as well as for two clones from each of the evolved lines. This experiment revealed no changes in any of the lines, except for one additional band (at ~500kb) that occurred exclusively in line S3 (Figure 9a). PFGE for chromosomes digested with the *Not*I restriction enzyme also revealed a single novel band (~180kb), which was again unique to line S3 (Figure 9b). Taken together, these observations suggest that high frequency copy number changes on a chromosomal scale are not rampant in our evolved lines, and the same arrangements do not occur across lines. All other distinguishable fragments in the gel, apart from the novel band of size ~180kb in line S3, were as predicted for a haploid yeast genome from a computational restriction digest of the genome with *Not*I. Additionally, chromosome-wide read coverage data from next generation sequencing (see below) supports the notion that there are no copy number changes involving large chromosome fragments (supplementary figure 4). However, a change in ploidy in the evolved lines that would affect entire chromosomes is not detectable by any of these two methods.

Gene Amplifications and Deletions from Next Generation Sequencing data

We next turned to smaller scale copy number changes that we could detect in our next generation sequencing data. To detect gene duplication and deletions, a segmentation algorithm (see supplementary methods) was used along with analysis of DNA breakpoints from the reads (see supplementary Methods). The results of both analyses are described in detail in the supplementary text.

We did not observe any large amplification or deletion in the genomes of any of the lines (and specifically in line S3) which could explain the appearance of novel bands in the pulsed field electrophoresis data from Figure 9. This observation, together with read coverage data of individual chromosomes (Supplementary figure 4) suggests that these novel bands were results of a non-duplicative translocation event in the line S3. However, for a haploid genome, a translocation event would displace one of the original bands in the PFGE, which we did not observe . Thus, such a translocation event is only consistent with the PFGE data if the affected chromosome occurs in more than one copy. In other words, it would be consistent with a ploidy change in line S3.

Ploidy of the Evolved Yeast Lines

Changes in ploidy of haploid yeast strains under salt stress have been

observed before (Gerstein *et al.*, 2006). Neither PFGE nor chromosome wide read coverage data would be able to detect such changes. To estimate the ploidy level of evolved lines in comparison to the ancestral strain, we grew ancestral strain and population samples of the evolved lines for 24hours at 30^{o}C and estimated the cell densities. We then isolated genomic DNA from a defined number of cells and quantified the DNA amount, which allowed us to calculate the DNA content per yeast cell for all the lines. We found that the DNA content per cell of lines S1 and S2 had increased by 78.7 percent and 85.4 percent, respectively, from that of the ancestral strain. For the line S3, the DNA content per cell was more than double that of the ancestral strain (119.91 percent increase from the ancestral strain)(Figure 10). These observations suggest that all the evolved lines have become massively aneuploid.

A consistent cell size increase during our experiment

Increases in ploidy are often associated with cell size increases. Our lines are no exception, as exemplified by the histogram of Figure 11a, which shows the distribution of cell diameters of the ancestral strain and the evolved line S2. This increase is also microscopically visible (Figure 11b). The mean cell diameter increased from 2.35µm (±0.70µm) to 6.73µm (±2.34µm). Along with a significant increase in the mean cell diameter (t-test, p-value <2.2×10^{-16}), the coefficient of variation (C_v), as defined by the ratio of standard deviation and mean, also increased significantly (t-test for distributions of C_v for samples drawn from two distributions, p-value<10^{-6}). In other words, not only did cells become larger during laboratory evolution, they also became more variable in size. Cell size changes in laboratory evolution experiments are not unprecedented. They have been observed in evolving *E. coli* populations (Lenski & Travisano, 1994; Lenski & Mongold, 2000; Philippe *et al.*, 2009), as well as in *Staphylococcus aureus* populations in NaCl medium (Vijaranakul *et al.*, 1995). However, effect of cell size on the survival and fitness are poorly understood.

Discussion

To investigate evolutionary adaptation to long-term osmotic stress, we evolved yeast cells in the laboratory for 300 generations in high salt (NaCl) medium. We observed that salt reduces the growth rate of our ancestral strain by 11 percent. Consequently, the final cell density after 24 hours of growth is approximately 3 times lower compared to cells grown in the same medium but without salt. Our three replicate evolved lines grow approximately 8 to 12 percent faster than the ancestral strain in high salt medium, and part of this increase reflects adaptation specific to salt.

We analyzed the gene expression levels in all the evolved lines as well as in the ancestral strain. Although there were many shared genes between the evolved lines and the ancestral strain that respond to salt in similar manner, we also observed multiple differentially expressed genes in the evolved lines compared to the ancestral strain. These differentially expressed genes can be divided into two categories. The first category comprises genes with changes in their basal expression level in the evolved lines compared to the ancestor (i.e., even in the absence of salt). The second category comprises genes regulated differently in response to salt in the evolved lines.

Multiple genes whose expression shows an *evolutionary* response in our experiments were also affected by hyperosmotic stress or salt stress in previous studies of the *physiological* stress response. Changed expression of many genes in the *physiological* stress response in general, and in the hyperosmotic stress response in particular is transient (Gasch *et al.*, 2000; Causton *et al.*, 2001), and may depend on the time and conditions in which it is measured. It is thus to be expected that different studies show limited comparability with respect to the identity of these genes.

Some of the genes whose expression evolved are known to be associated with the stress response. For example, two genes, *CTT1* and *GAC1,* showed an increase in basal expression in the evolved lines compared with the ancestral strain. Both these genes were also up-regulated in response to salt in our ancestral strain. *CTT1* encodes for a cytosolic catalase and protects the cell from oxidative damage by reactive oxygen species (Jamieson, 1998; Lushchak & Gospodaryov, 2005; Herrero *et al.*, 2008). This gene has been shown to be up-regulated in four previous studies of *physiological* stress response and salt stress response (Gasch *et al.*, 2000; Rep *et al.*, 2000; Causton *et al.*, 2001; Yale & Bohnert, 2001). The gene *GAC1* is associated with glycogen synthesis (Wu *et al.*, 2001) and is also induced during osmo-adaptation (Posas *et al.*, 2000). Both these genes contain stress responsive elements (STRE) in their promoters (Schuller *et al.*, 1994; Moskvina *et al.*, 1998) and are known to be activated under various stress conditions. A third gene, *CUP1-1*, showed increased induction in the evolved lines and also was up-regulated in response to NaCl in our ancestral strain. This gene was also shown to be up-regulated in one previous study of *physiological* stress response (Yale & Bohnert, 2001). Yet another gene, *HLR1,* showed increased repression in the evolved lines, and was down-regulated in the ancestral strain in NaCl medium. This gene encodes a protein involved in maintaining cell wall composition and is also involved in response to osmotic stress (Alonso-Monge *et al.*, 2001).

There are three main *physiological* components of the salt stress response. They affect adaptation time to salt, growth rate in salt, and efficiency of growth in salt (Warringer *et al.*, 2003). In our experiments, *evolutionary* adaptation to salt must affect one or more of these three component, because the viability itself of yeast cells does not change between the ancestral strain and the evolved lines. There are various ways in which these components could change during evolution. Firstly, some of the genes whose basal expression changes might be directly involved in the initial adaptation to salt. Increasing or decreasing the basal expression of those genes to the level needed for salt adaptation, might enable the cells respond to salt much more quickly as opposed to changing their expression state. For example, some ion transporter genes (e.g. *FRE5)* have an increased basal expression in the evolved lines in our experiment. Higher levels of these transporter proteins at the initial stages of the salt stress response would help cells achieve ion homeostasis much more quickly. Secondly, some of the differentially expressed genes might not be directly related to the salt stress response but encode transcription factors that controls salt stress response genes. For example, the transcription factor *MSN4*, that controls many stress response genes, has increased basal expression levels in our evolved yeast lines. Higher levels of this

transcription factor during the initial adaptation phase could ensure faster induction of the genes required for salt stress response. Thirdly, there are genes that show evolutionary change in regulation in the evolved lines. Higher level of induction or repression of these genes might increase the growth rate and/or efficiency of growth in salt medium.

We found 16 genes that gained regulation (new induction and new repression) in the evolution experiment, suggesting that new ways of salt stress adaptation could also arise during evolution. However, we also observed loss of regulation, at least to some extent, for five genes (genes with reduced induction and reduced repression) in the evolved lines. Because we performed our evolution experiment under constant environmental stress, losing regulation might be advantageous for evolutionary adaptation to salt stress. If the affected genes are involved in adaptation to multiple stresses, losing regulation could actually be beneficial both under constant or fluctuating environmental stressors. However, if the affected genes are specific for that particular stress, a loss in regulation may also have a cost, because cells would become physiologically less flexible under fluctuating environmental stresses. Among the five genes with loss of regulation, one gene, *HSP30,* can be induced by several stresses (Piper *et al.*, 1997). However, the mRNA level of this gene was shown to be diminished in NaCl medium by Rep *et al* (Rep *et al.*, 2000).

The hyperosmotic and salt stress responses are complex and influenced by many genes. It is an open question whether a small number of genetic changes with large effects could dramatically increase salt stress resistance, and thus explain the fitness increase in our evolved lines. To find out, we also sequenced the ancestral strain and population samples of the evolved lines using deep sequencing with genome coverages between 10- and 50-fold to identify whether any high frequency polymorphisms arose in the evolved lines during our evolution experiment. For two reasons, we chose deep population sequencing over clone sequencing. First, population sequencing gives a comprehensive view of polymorphisms in a population. Second, it can also permit estimation of polymorphism frequencies, although any such estimate would be more qualitative than quantitative at our sequence coverage. At the very least, population sequencing can detect high frequency polymorphisms. Such polymorphisms are of the greatest interest to us, because they would correspond to adaptive mutations with strong fitness effects that rose to high frequencies. We note that the population sizes in our experiment are so large that no neutral polymorphisms would be expected to rise to high frequencies during the experiment's short duration except through hitchhiking with an advantageous mutation (effective population size in our experiment is $>2 \times 10^6$). (Clone sequencing can detect low frequency polymorphisms, but for a heterogeneous population, this method requires sequencing of many clones, and is thus currently prohibitively costly.)

Only one of our evolved lines (S2) contained a high frequency single nucleotide polymorphism (SNP). This SNP occurs in more than 50 percent of the population, and causes an amino acid substitution in the protein encoded by the *MOT2* gene. When introduced into the ancestral strain, this mutation causes a fitness increase

that explains 25.3 percent of the increase in fitness *w* in the evolved strain. However, the mutation causes a fitness increase also in the absence of salt, which means that its effects do not reflect a specific adaptation to salt.

Multiple genetic changes of low population frequency could be present in our evolved lines, and these changes might explain a part of the fitness increase we see. Such low frequency SNPs might be present for several reasons. First, individual mutations may confer only modest fitness benefits and thus increase in frequency slowly. Second, some mutations with very strong fitness effects may have occurred, but late in the experiment, and thus might simply not have had enough time to rise to high frequency..Third, the fitness effect of alleles might be determined by epistatic interactions with other mutations elsewhere in the genome (Elena & Lenski, 1997; Elena & Lenski, 2001; Morgan & Feldman, 2001). Eventually genotypes with such alleles might rise to high frequency, but at time scales much longer than those of our experiment, because multiple mutations might have to occur before any one epistatic combination with strong benefits arises. Finally, and perhaps most importantly, there could be clonal interference between multiple beneficial polymorphisms in the population and this might prevent any particular polymorphism from rising to high frequency in the population (Kao & Sherlock, 2008).

Similar to our analysis on SNPs, our analysis on copy number variations, both based on PFGE and next generation sequencing, did not reveal any large scale copy number changes shared across strains. However, we observed ploidy increases in all the evolved lines, and this could be the reason behind increase in cell size of these lines.

Evidence from plants suggests that an increase in ploidy and the resultant increase in cell size can be advantageous under salt-stressed conditions. For example, within a species, plants with higher ploidy can cope with salt stress better than plants with lower ploidy (Saleh *et al.*, 2008). Polyploid plants have higher water content and lower osmotic pressure than diploid plant (Noggle, 1946). Their increased water content is due to a decrease in the surface-to-volume ratio of cells (Stebbins, 1950). In addition, any water content decrease due to high salinity is smaller in polyploid plants than that in diploid plants (Tal & Gardi, 1976). Gerstein *et al.* observed that initially haploid yeast lines increased genome size significantly faster under salt stress than in unstressed condition, suggesting an advantage for higher ploidy or increased cell size also in yeast (Gerstein *et al.*, 2006).

Changes in ploidy could help yeast cells adapt faster physiologically, for example by affecting expression of genes important for salt stress tolerance. Could all the changes in gene expression that we observed in our experiment be caused solely by ploidy changes or by the concomitant increase in cell size? To find out, we compared the set of genes that changed expression in our evolved lines with the set of genes that are known to change expression after cell size increases (Wu *et al.*, 2010) and ploidy changes (Galitski *et al.*, 1999). Remarkably, only three genes that evolve changed expression in our experiment (out of 144 differentially expressed genes in our dataset) are among the genes affected by cell size increase or ploidy changes. Two of these genes showed increased basal expression in our experiment. One of them, YIL169C,

was also observed to be induced with increase in cell size (Wu *et al.*, 2010). The other gene, *COS8,* was observed to change expression with a change in ploidy level (Galitski *et al.*, 1999). The third gene, YLR042C, showed a basal decrease in expression in our evolved lines. It was also observed to be repressed in cells with increased size (Wu *et al.*, 2010).

Overall, the vast majority of changes that we observe in gene expression cannot be caused by changed ploidy or cell size. Because our whole genome sequencing analysis revealed no small genetic changes of strong phenotypic effects, the gene expression changes we observe could be caused by multiple genetic changes of modest individual effects. Some of these changes might affect a gene's expression in *cis*, others in *trans*, for example through changes in the amino acid sequence of transcriptional regulators (Wittkopp *et al.*, 2004; Emerson & Li, 2010). In addition, some expression changes may be caused by epigenetic change (e.g., changes in DNA methylation) or alterations in cellular memory (Turner, 2002; Ringrose & Paro, 2004; Zhang *et al.*, 2005; Zacharioudakis *et al.*, 2007).

We found several genes with modest changes in expression level in our evolved lines, and no gene with very strong expression change. Our observations stand in contrast to the only laboratory evolution study we know of that investigated evolutionary adaptation to a stressor. The study asked how *E. coli* cells adapt to heat stress. It observed very high level of evolutionary expression change in two proteins, GroEL and GroES, that are known heat shock genes (Rudolph *et al.*, 2010). The reason for this difference to our work is probably that adaptation to salt stress requires several molecular functions simultaneously, namely ionic detoxification to achieve ionic equilibrium, maintaining cellular water activity, and osmolyte synthesis. Each of the evolutionarily altered genes in our experiment may perform or control some of these functions and thus, may have only a modest individual fitness contribution when differentially expressed. In addition, gene interactions (Phillips 2008; He *et al.*, 2010) could also contribute towards fitness increase. This scenario resembles recent observations in genome wide association studies (GWAS) for complex human traits and diseases, where researchers have observed many genetic variants with modest effects on a phenotype (Frayling, 2007; Barrett *et al.*, 2008; Cooper *et al.*, 2008; Visscher, 2008; Zeggini *et al.*, 2008; Hindorff *et al.*, 2009; Manolio *et al.*, 2009; Visscher & Montgomery, 2009; Park *et al.*, 2010; Yang *et al.*, 2010).

Taken together, adaptation to salt stress is associated with DNA content increase and gene expression change in all the three lines, one high frequency mutation in one of the evolved lines, and one chromosomal rearrangement in another. The increase in DNA content in all the lines is massive and previous studies suggest that the change in ploidy might be beneficial for the lines in salt. In contrast, there are 144 genes in our study that show modest change in their expression levels. This observation suggests that tinkering with expression levels of several genes might be a useful mechanism to respond to a complex, polygenic trait rather than altering expression of few genes to very high levels. Such tinkering might also reflect cellular constraints on evolution. For example, changing expression of a gene to very high levels or suppressing a gene completely might increase fitness of the yeast cells in salt medium.

However, changing expression of a gene to very high levels or suppressing a gene completely might affect the capability of the cells to grow in other media. Since we do not find high frequency genetic changes that can explain expression changes of the genes, it is possible that these are caused by multiple genetic changes of low frequency (that might vary from one subpopulation to other) or by epigenetic changes. These results also suggest that cells in our populations perhaps employ several strategies in combination for adaptation to salt stress rather than following a single path to adaptation. It is possible that such genetic heterogeneity might render the populations to be more robust in the long term. If some of the genetic factors contributing to the salt stress adaptation are lost, the fitness of the population would not fall drastically. Also, such diverse populations might be less vulnerable against other environmental fluctuations which might co-occur with saline stress in natural environment. Although individual contributions of each of these genetic mechanisms to adaptation remain unclear, our study provides a stepping stone for asking further questions on *evolutionary* adaptation of yeast to salt stress.

Methods

Strains and media.

All laboratory evolution experiments started from the same clone of haploid yeast strain BY4741, which is referred to as the *ancestral* strain. The 3 replicate yeast lines evolved in NaCl are referred to as lines S1, S2 and S3. The growth rates of the evolved lines as well as the ancestral strain were measured relative to a BY4741 strain in which the *CWP2* gene was GFP-tagged (termed as the *reference* strain). For serial transfers, cells were cultured in YP and 2% galactose (YPG) and YPG supplemented with 0.5 M NaCl (YPGN).

Serial transfer

Six parallel serial transfer experiments were started from one single clone of the ancestral strain. In each parallel experiment, 50 ml yeast culture was grown for at 30^{o}C. Every 24 hours, 50µl of grown culture was transferred into fresh culture medium; 30 such transfer cycles were carried out for a total of approximately 300 generations (Each transfer cycle involved approximately $\log_2 1000 \approx 10$ cell generations). In three of the parallel experiments, the culture medium was YPG , whereas in the other three parallel experiments, the medium was YPGN.

Viability assays

To estimate cell viabilities, cultures of growing yeast cells were sampled after 16 hours (during exponential growth phase), as well as after 24 hours (during stationary phase), diluted and plated. The plates were incubated at 30^{o}C for 5 days and the number of colonies were counted. The relative viabilities of both the ancestral strain and the evolved lines were estimated from the ratio of colonies forming on agar plates containing YPG+ 0.5M NaCl to that of plates containing only YPG. All the measurements were carried out in three biological replicates.

Competition assays

To compare growth rates of the evolved lines with that of the ancestral strain, competition assays were carried out in triplicates using fluorescence activated cell sorting (FACS). Cells from frozen glycerol stocks were grown overnight in 4ml YPD medium (30^{o}C, 220rpm) until late logarithmic phase (<1.5x10^{8} cells/ml). For the competition assay, approximately equal cell numbers of the reference strain and of the competing strain were mixed and grown for 24 hours at 30^{o}C. The relative cell numbers at the beginning and at the end of the competition experiment were determined using FACS (supplementary methods) and growth rate differences were estimated as described in supplementary methods. For calculation of fitness, the relative cell numbers at the beginning of the assay were taken into consideration.

Whole genome transcriptome analysis

The mRNA expression levels in the ancestral strain and in the evolved lines were analyzed using a GeneChip Yeast Genome 2.0 Array (Affymetrix). Equal number of cells from the ancestral strain and the evolved lines were grown in YPG medium for 16 hours. The cells were then either induced with 0.5M NaCl or grown uninduced for 20 further minutes. The microarray analysis was carried out for 2 replicates each for the ancestral strain in YPG and YPGN (4 in total) and for 4 replicate population samples (1 for S1,2 for S2 and 1 for S3) each in YPG and YPGN for the S lines (8 in total). "Shared" genes, genes that respond to NaCl in a similar manner between the ancestral strain and the evolved lines, with significant up-regulation or down-regulation were identified using a t-test at p=0.05, False Discovery Rate(FDR)<10% and $|\log_2(\text{fold change})| \geq 1$.

Genes with changes in basal expression or change in regulation were identified based on two Z scores Z_r and Z_b (see supplementary methods). Genes whose absolute Z-scores exceeded a value of 1.5 were considered to be differentially expressed. The differentially expressed genes were then grouped into different classes using the CYGD functional classification for yeast genes (Güldener *et al.*, 2005).

Whole Genome Sequencing and SNP identification

The ancestral strain, as well as population samples of the NaCl evolved lines at generation 300 were sequenced at approximately 10X coverage using next generation pyrosequencing (Margulies *et al.*, 2005) (genome sequencer FLX LR system, Roche). The line S3 was further sequenced to a total of ~50X coverage. Candidate SNPs and indels were identified using blastn (Altschul *et al.*, 1990) followed by MUSCLE (Edgar, 2004) based on the criteria described in supplementary methods. PCR sequencing was done to confirm candidate SNPs and indels.

Pulsed-field gel electrophoresis (PFGE)

To identify large scale chromosomal rearrangements, two clones from each of the evolved lines (S1, S2 and S3) and the ancestral strain were analyzed by PFGE. Agarose plugs were prepared with the CHEF Yeast Genomic DNA Plug Kit (Bio-Rad) according to the manufacturer's protocol. For restriction enzyme digestion, agarose

plugs were incubated with NotI restriction enzyme for 16hrs at 37^{o}C mixture in an appropriate restriction buffer. The digested or undigested plugs were then loaded into the wells of 1% agarose gels. Electrophoresis was carried out in a CHEF-DRIII system (Bio-Rad). The gels were stained using ethidium bromide (EtBr) and photographed.

Gene Amplification and Deletions from Next Generation Sequencing data

In addition to identifying very short indels within individual reads, larger gene duplications and deletions in the evolved lines, compared to the ancestral strain, were identified using read coverage information from the sequencing data. Furthermore, DNA breakpoint analysis was performed to confirm those candidate copy number changes.

Ploidy determination of the evolved lines

To estimate the ploidy level of the evolved lines in comparison to the ancestral strain, the ancestral strain and population samples of the evolved lines were grown for 24 hours at 30^{o}C. Genomic DNA was isolated from a defined number of cells following the protocol described in (Sambrook & Russell, 2001, Chapter 6).

Cell size measurement

Population samples of ancestral and evolved cells were grown in YPG medium up to mid-log phase. The cell density was adjusted to be the same for all the samples (~1x10^{7}cells/ml). 20ul of cells were then loaded onto disposable counting chambers and the cell sizes, were measured using a Cellometer M10 (Nexcelom Biosciences), following the manufacturer's protocol.

Acknowledgements

We would like to acknowledge the help of the Functional Genomics Center Zurich for carrying out next generation sequencing and microarray experiments. AW acknowledges support through SNF grants 315200-116814, 315200-119697, and 315230-129708, as well as through the YeastX project of SystemsX.ch, and the University Research Priority Program in systems biology at the University of Zurich.

References

Adams, J. & Oeller, P.W. 1986. Structure of evolving populations of *Saccharomyces cerevisiae*: Adaptive changes are frequently associated with sequence alterations involving mobile elements belonging to the Ty family. *Proc. Natl. Acad. Sci. USA* **83**: 7124-7127.

Adams, J., Puskas-Rozsa, S., Simlar, J. & Wilke, C.M. 1992. Adaptation and major chromosomal changes in populations of *Saccharomyces cerevisiae*. *Curr. Genet.* **22**: 13-19.

Alonso-Monge, R., Real, E., Wojda, I., Bebelman, J.P., Mager, W.H. & Siderius, M. 2001. Hyperosmotic stress response and regulation of cell wall integrity in *Saccharomyces cerevisiae* share common functional aspects. *Mol. Microbiol.* **41**: 717-

730.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**: 403-410.

Apse, M.P., Aharon, G.S., Snedden, W.A. & Blumwald, E. 1999. Salt Tolerance Conferred by Overexpression of a Vacuolar Na +/H+ Antiport in *Arabidopsis*. *Science* **285**: 1256-1258.

Attfield, P.V. 1997. Stress Tolerance: The key to effective strains of industrial baker's yeast. *Nat. Biotech.* **15**: 1351-1357.

Badarinarayana, V., Chiang, Y.C. & Denis, C.L. 2000. Functional interaction of CCR4-NOT proteins with TATAA-binding protein (TBP) and its associated factors in yeast. *Genetics* **155**: 1045-1054.

Barrett, J.C., Hansoul, S., Nicolae, D.L., Cho, J.H., Duerr, R.H., Rioux, J.D. *et al.* 2008. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. *Nat. Genet.* **40**: 955-962.

Betz, C., Zajonc, D., Moll, M. & Schweizer, E. 2002. ISC1-encoded inositol phosphosphingolipid phospholipase C is involved in Na+/Li+ halotolerance of *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **269**: 4033-4039.

Blanc, V.M. & Adams, J. 2003. Evolution in *Saccharomyces cerevisiae*: Identification of Mutations Increasing Fitness in Laboratory Populations. *Genetics* **165**: 975-983.

Bressan, R.A., Hasegawa, P.M. & Pardo J.M. 1998. Plants use calcium to resolve salt stress. *Trends Plant Sci.* **3**: 411-412.

Brewster, J.L., de Valoir, T., Dwyer, N.D., Winter, E. & Gustin, M.C. 1993. An Osmosensing Signal Transduction Pathway in Yeast. *Science* **259**: 1760-1763.

Cade, R.M. & Errede, B. 1994. MOT2 encodes a negative regulator of gene expression that affects basal expression of pheromone-responsive genes in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **14**: 3139-3149.

Causton, H.C., Ren, B., Koh, S.S., Harbison, C.T., Kanin, E., Jennings, E.G. *et al.* 2001. Remodeling of Yeast Genome Expression in Response to Environmental Changes. *Mol. Biol. Cell* **2**: 323-337.

Cooper, J.A. 1994. MAP KINASE PATHWAYS: Straight and narrow or tortuous and intersecting? *Curr. Biol.* **4**: 1118-1121.

836 Cooper, J.D., Smyth, D.J., Smiles, A.M., Plagnol, V., Walker, N.M., Allen, J.E. *et al.*
837 2008. Meta-analysis of genome-wide association study data identifies additional type 1
838 diabetes risk loci. *Nat. Genet.* **40**: 1399-1401.
839
840 Denis, C.L., Chiang, Y.C., Cui, Y. & Chen, J. 2001. Genetic evidence supports a role for
841 the yeast CCR4-NOT complex in transcriptional elongation. *Genetics* **158**: 627-634.
842
843 Dihazi, H., Kessler, R. & Eschrich, K. 2004. High Osmolarity Glycerol (HOG) Pathway-
844 induced Phosphorylation and Activation of 6-Phosphofructo-2-kinase Are Essential for
845 Glycerol Accumulation and Yeast Cell Proliferation under Hyperosmotic Stress. *J. Biol.*
846 *Chem.* **279**: 23961-23968.
847
848 Dunham, M.J., Badrane, H., Ferea, T., Adams, J., Brown, P.O., Rosenzweig, F. *et al.*
849 2002. Characteristic genome rearrangements in experimental evolution of
850 *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **99**: 16144-16149.
851
852 Edgar, R.C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high
853 throughput. Nucleic Acids Res. 32:1792-1797.
854
855 Elena, S.F. & Lenski, R.E. 1997. Test of synergistic interactions among deleterious
856 mutations in bacteria. *Nature* **390**: 395-398.
857
858 Elena, S.F. & Lenski, R.E. 2001. Epistasis between new mutations and genetic
859 background and a test of genetic canalization. *Evolution* **55**: 1746-1752.
860
861 Emerson, J.J. & Li, W-H. 2010. The genetic basis of evolutionary change in gene
862 expression levels. *Phil. Trans. R. Soc. B* **365**: 2581-2590.
863
864 Errede, B. & Levin, D.E. 1993. A conserved kinase cascade for MAP kinase activation
865 in yeast. *Curr. Opin. Cell Biol.* **5**: 254-260.
866
867 Espinosa-Ruiz, A., Belles, J.M., Serrano, R. & Culianez-Macla, V. 1999. *Arabidopsis*
868 *thaliana* AtHAL3: A flavoprotein related to salt and osmotic tolerance and plant growth.
869 *Plant J.* **20**: 529-539.
870
871 Ferea, T.L., Botstein, D., Brown, P.O. & Rosenzweig, R.F. 1999. Systematic changes in
872 gene expression patterns following adaptive evolution in yeast. *Proc. Natl. Acad. Sci.*
873 *USA*. 96: 9721-9726.
874
875 Ferrando, A., Kron, S.J., Rios, G., Fink, G.R. & Serrano, R. 1995. Regulation of cation
876 transport in *Saccharomyces cerevisiae* by the salt tolerance gene HAL3. *Mol. Cell. Biol.*
877 **15**: 5470-5481.
878
879 Frayling, T.M. 2007. Genome-wide association studies provide new insights into type 2

880 diabetes aetiology. *Nat. Rev. Genet.* **8**: 657-662.
881
882 Galcheva-Gargova, Z., Derijard, B., Wu, I.H. & Davis, R.J. 1994. An osmosensing signal
883 transduction pathway in mammalian cells. *Science* **265:** 806-808**.**
884
885 Galitski, T., Saldanha, A.J., Styles, C.A., Lander, E.S. & Fink, G.R. 1999. Ploidy
886 regulation of gene expression. *Science* **285: 251-254.**
887
888 Ganster, R.W., McCartney, R.R. & Schmidt, M.C. 1998. Identification of a Calcineurin-
889 Independent Pathway Required for Sodium Ion Stress Response in *Saccharomyces*
890 *cerevisiae*. *Genetics* **150**: 31-42.
891
892 Garciadeblas, B., Rubio, F., Quintero, F.J., Bañuelos, M.A., Haro, R. & Rodríguez-
893 Navarro, A. 1993. Differential expression of two genes encoding isoforms of the ATPase
894 involved in sodium efflux in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **236**: 363-368.
895
896 Gasch, A.P., Spellman, P.T., Kao, C.M., Carmel-Harel, O., Eisen, M.B., Storz, G. *et al.*
897 2000. Genomic Expression Programs in the Response of Yeast Cells to Environmental
898 Changes. *Mol. Biol. Cell* **11**: 4241-4257.
899
900 Gaxiola, R., de Larrinoa, I.F., Villalba, J.M. & Serrano, R. 1992. A novel and conserved
901 salt-induced protein is an important determinant of salt tolerance in yeast. *EMBO J.* **11**:
902 3157-3164.
903
904 Gaxiola, R.A., Rao, R., Sherman, A., Grisafi, P., Alper, S.L. & Fink, G.R. 1999. The
905 *Arabidopsis thaliana* proton transporters, AtNhx1 and Avp1, can function in cation
906 detoxification in yeast. *Proc. Natl. Acad. Sci. USA* **96**: 1480-1485.
907
908 Gerstein, A.C., Chun, H.J.E., Grant, A. & Otto, S.P. 2006. Genomic convergence toward
909 diploidy in *Saccharomyces cerevisiae*. *PLoS Genet.* **2**: e145.
910
911 Gisbert, C., Rus, A.M., Bolarín, M.C., Lopez-Coronado, J.M., Arrillaga, I., Montesinos,
912 C. *et al.* 2000. The Yeast HAL1 Gene Improves Salt Tolerance of Transgenic Tomato.
913 *Plant Physiol.* **123**: 393-402.
914
915 Goossens, A., Forment, J. & Serrano, R. 2002. Involvement of Nst1p/YNL091w and
916 Msl1p, a U2B" splicing factor, in *Saccharomyces cerevisiae* salt tolerance. *Yeast* **19**:
917 193-202.
918
919 Gorner, W., Durchschlag, E., Martinez-Pastor, M.T., Estruch, F., Ammerer, G. &
920 Hamilton, B. *et al.* 1998. Nuclear localization of the C2H2 zinc finger protein Msn2p is
921 regulated by stress and protein kinase A activity. *Genes Dev.* **12**: 586-597.
922
923 Gresham, D., Desai, M.M., Tucker, C.M., Jenq, H.T., Pai, D.A., Ward, A. *et al.* 2008.

924 The Repertoire and Dynamics of Evolutionary Adaptations to Controlled Nutrient-
925 Limited Environments in Yeast. *PLoS Genet.* **4**: e1000303.
926
927 Güldener, U., Münsterkötter, M., Kastenmüller, G., Strack, N., van Helden, J., Lemer, C.
928 *et al.* 2005. CYGD: the Comprehensive Yeast Genome Database. *Nucl. Acids Res.* **33**:
929 D364-D368.
930
931 Han, J., Lee, J.D., Bibbs, L. & Ulevitch, R.J. 1994. A MAP kinase targeted by endotoxin
932 and hyperosmolarity in mammalian cells. *Science* **265:** 808-811**.**
933
934 Haro, R., Garciadeblas, B. & Rodríguez-Navarro, A. 1991. A novel P-type ATPase from
935 yeast involved in sodium transport. *FEBS Lett.* **291**: 189-191.
936
937 Hasegawa, P.M. & Bressan, R.A. 2000. Plant Cellular and Molecular Responses to
938 High Salinity. *Annu. Rev. Plant. Physiol. Plant. Mol. Biol.* **51**: 463-499.
939
940 He, X., Qian, W., Wang, Z., Li, Y. & Zhang, J. 2010. Prevalent positive epistasis in
941 *Escherichia coli* and *Saccharomyces cerevisiae* metabolic networks. *Nat. Genet.* **42**:
942 272-276.
943
944 Heath, V.L., Shaw, S.L., Roy, S. & Cyert, M.S. 2004. Hph1p and Hph2p, Novel
945 Components of Calcineurin-Mediated Stress Responses in *Saccharomyces cerevisiae*.
946 *Eukaryotic Cell* **3**: 695-704
947
948 Herrero, E., Ros, J., Bellí, G. & Cabiscol, E. 2008. Redox control and oxidative stress in
949 yeast cells. *Biochim. Biophys. Acta* **1780**: 1217-1235.
950
951 Hindorff, L.A., Sethupathy, P., Junkins, H.A., Ramos, E.M., Mehta, J.P., Collins, F.S. *et*
952 *al.* 2009. Potential etiologic and functional implications of genome-wide association loci
953 for human diseases and traits. *Proc. Natl. Acad. Sci.* USA **106**: 9362–9367.
954
955 Hirata, Y., Andoh, T., Asahara, T. & Kikuchi, A. 2003. Yeast Glycogen Synthase Kinase-
956 3 Activates Msn2p-dependent Transcription of Stress Responsive Genes. *Mol. Biol.*
957 *Cell.* **14**: 302-312.
958
959 Hirt, H. 1997. Multiple roles of MAP kinases in plant signal transduction. *Trends Plant*
960 *Sci.* **2**: 11-15.
961
962 Hohmann, S. 2002. Osmotic Stress Signaling and Osmoadaptation in Yeasts. *Micro.*
963 *Mol. Biol. Rev.* **66**: 300–372.
964
965 Hughes, T.R., Roberts, C.J., Dai, H., Jones, A.R., Meyer, M.R., Slade, D. *et al.* 2000.
966 Widespread aneuploidy revealed by DNA microarray expression profiling. *Nat. Genet.*
967 **25**: 333-337.

Jamieson, D.J. 1998. Oxidative stress responses of the yeast Saccharomyces cerevisiae. *Yeast* **14**: 1511-1527.

Jonak, C., Kiegerl, S., Ligterink, W., Barker, P.J., Huskisson, N.S. & Hirt, H. 1996. Stress signaling in plants: A mitogen-activated protein kinase pathway is activated by cold and drought. *Proc. Natl. Acad. Sci. USA* **93**: 11274-11279.

Jonak, C., Ligterink, W. & Hirt, H. 1999. MAP kinases in plant signal transduction. *Cell. Mol. Life Sci.* **55**: 204-213.

Kao, K.C. & Sherlock, G. 2008. Molecular characterization of clonal interference during adaptive evolution in asexual populations of *Saccharomyces cerevisiae. Nat. Genet.* **40**: 1499-1504.

Kosako, H., Nishida, E. & Gotoh, Y. 1993. cDNA cloning of MAP kinase kinase reveals kinase cascade pathways in yeasts to vertebrates. *EMBO J.* **12**: 787-794.

Kovtun, Y., Chiu, W-L., Tena, G., Sheen, J. 2000. Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. *Proc. Natl. Acad. Sci. USA* **97**: 2940-2945.

Kyriakis, J.M. & Avruch, J. 2001. Mammalian Mitogen-Activated Protein Kinase Signal Transduction Pathways Activated by Stress and Inflammation. *Physiol. Rev.* **81**: 807-869.

Lee, J.H., Montagu, M.V. & Verbruggen, N. 1999. A highly conserved kinase is an essential component for stress tolerance in yeast and plant cells. *Proc. Natl. Acad. Sci. USA* **96**: 5873-5877.

Lenski, R.E. & Travisano, M. 1994. Dynamics of adaptation and diversification: A 10,000-generation experiment with bacterial populations. *Proc. Natl. Acad. Sci. USA* **91**: 6808-6814.

Lenski, R.E. & Mongold, J.A. 2000. Cell size, shape, and fitness in evolving populations of bacteria. In: *Scaling in Biology* (J. H. Brown & G. B. West, eds), pp. 221-235, Oxford University Press.

Liu, H.Y., Badarinarayana, V., Audino, D.C., Rappsilber, J., Mann, M. & Denis, C.L. 1998. The NOT proteins are part of the CCR4 transcriptional complex and affect gene expression both positively and negatively. *EMBO J.* **17**: 1096-1106.

Lushchak, V.I. & Gospodaryov, D.V. 2005. Catalases protect cellular proteins from oxidative modification in *Saccharomyces cerevisiae*. *Cell Biol. Int.* **29**: 187-192.

Maathuis, F.J.M. & Amtmann, A. 1999. K+ Nutrition and Na+ Toxicity : The Basis of Cellular K+/Na+ Ratios. *Annals of Bot.* **84**: 123-133.

Mai, B. & Breeden, L. 1997. Xbp1, a Stress-Induced Transcriptional Repressor of the *Saccharomyces cerevisiae* Swi4/Mbp1 Family. *Mol. Cell. Biol.* **17**: 6491-6501.

Manolio, T.A., Collins, F.S., Cox, N.J., Goldstein, D.B., Hindorff, L.A., Hunter, D.J. *et al.* 2009. Finding the missing heritability of complex diseases. *Nature* **461**: 747-753.

Margulies, M., Egholm, M., Altman, W.E., Attiya, S., Bader, J.S., Bemben, L.A. *et al.* 2005. Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **437**: 376-380.

Martinez-Pastor, M.T., Marchler, G., Schuller, C., Marchler-Bauer, A., Ruis, H. & Estruch, F. 1996. The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress-response element (STRE). *EMBO J.* **15**: 2227-2235.

Mendizabal, I., Rios, G., Mulet, J.M., Serrano, R. & de Larrinoa, I.F. 1998. Yeast putative transcription factors involved in salt tolerance. *FEBS Lett.* **425**: 323-328.

Mendoza, I., Rubioli, F., Rodriguez-Navarroli, A. & Pardo, J.M. 1994. The Protein Phosphatase Calcineurin Is Essential for NaCl Tolerance of *Saccharomyces cerevisiae*. *J. Biol. Chem.* 269: 8792-8796.

Mersman, D.P., Du, H.N., Fingerman, I.M., South, P.F. & Briggs, S.D. 2009. Polyubiquitination of the demethylase Jhd2 controls histone methylation and gene expression. *Genes Dev.* **23**: 951-962.

Morgan, L.W. & Feldman, J.F. 2001. Epistatic and Synergistic Interactions Between Circadian Clock Mutations in *Neurospora crassa*. *Genetics* **159**: 537-543.

Moskvina, E., Schuller, C., Maurer, C.T.C., Mager, W.H. & Ruis, H. 1998. A Search in the Genome of *Saccharomyces cerevisiae* for Genes Regulated via Stress Response Elements. *Yeast* **14**: 1041-1050.

Mulet, J.M., Leube, M.P., Kron, S.J., Rios, G., Fink, G.R. & Serrano, R. 1999. A novel mechanism of ion homeostasis and salt tolerance in yeast: the Hal4 and Hal5 protein kinases modulate the Trk1-Trk2 potassium transporter. *Mol. Cell. Biol.* **19**: 3328-3337.

Mukhopadhyay, A., He, Z., Alm, E.J., Arkin, A.P., Baidoo, E.E., Borglin, S.C. *et al.* 2006: Salt Stress in *Desulfovibrio vulgaris* Hildenborough: an Integrated Genomics Approach. *J. Bacteriol.* **188**: 4068-4078.

Murguía, J.R., Bellés, J.M. & Serrano, R. 1996. The yeast HAL2 nucleotidase is an in vivo target of salt toxicity. *J. Biol. Chem.* **271**: 29029-29033.

Neiman, A.M. 1993. Conservation and reiteration of a kinase cascade. *Trends Genet.* 9: 390-394.

Neiman, A.M., Stevenson, B.J., Xu, H-P., Sprague, Jr. G.F., Herskowitz, I., Wigler, M. et al. 1993. Functional Homology of Protein Kinases Required for Sexual Differentiation in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* Suggests a Conserved Signal Transduction Module in Eukaryotic Organisms. *Mol. Biol. Cell* **4**: 107-120.

Noggle, R. 1946.The physiology of polyploidy in plants. I. Review of the literature. *Lloydia* **9**; 153-173.

Nishida, E. & Gotoh, Y. 1993. The MAP kinase cascade is essential for diverse signal transduction pathways. Trends Biochem. Sci. 18: 128-131.

Ostrander, D.B. & Gorman, J.A. 1999. The Extracellular Domain of the *Saccharomyces cerevisiae* Sln1p Membrane Osmolarity Sensor Is Necessary for Kinase Activity. *J. Bacteriol.* **181**: 2527-2534.

Panaretou, B. & Piper, P.W. 1992. The plasma membrane of yeast acquires a novel heat-shock protein (hsp30) and displays a decline in proton-pumping ATPase levels in response to both heat shock and the entry to stationary phase. *Eur. J. Biochem*. **206**: 635-640.

Panasenko, O., Landrieux, E., Feuermann, M., Finka, A., Paquet, N. & Collart, M.A. 2006. The yeast Ccr4-Not complex controls ubiquitination of the nascent-associated polypeptide (NAC-EGD) complex. *J. Biol. Chem.* **281**: 31389-31398.

Pardo, J.M., Reddy, M.P., Yang, S., Maggio, A., Huh, G-H., Matsumoto, T. *et al.* 1998. Stress signaling through Ca^{2+}/ calmodulin-dependent protein phosphatase calcineurin mediates salt adaptation in plants. *Proc. Natl. Acad. Sci. USA* **95**: 9681-9686.

Park, J.H., Wacholder, S., Gail, M.H., Peters, U., Jacobs, K.B., Chanock, S.J. *et al.* 2010. Estimation of effect size distribution from genome-wide association studies and implications for future discoveries. *Nat. Genet.* **42**: 570-575.

Pelech, S.L. & Sanghera, J.S. 1992. Mitogen-activated protein kinases: versatile transducers for cell signaling. *Trends Biochem. Sci.* **17**: 233-238.

Philippe, N., Pelosi, L., Lenski, R.E. & Schneider, D. 2009. Evolution of Penicillin-Binding Protein 2 Concentration and Cell Shape during a Long-Term Experiment with

Escherichia coli. *J. Bacteriol.* **191**: 909-921.

Phillips, P.C. 2008. Epistasis - the essential role of gene interactions in the structure and evolution of genetic systems. *Nat. Rev. Genet.* **9**: 855-867.

Piper, P.W., Ortiz-Calderon, C., Holyoak, C., Coote, P. & Cole, M. 1997. Hsp30, the integral plasma membrane heat shock protein of *Saccharomyces cerevisiae*, is a stress-inducible regulator of plasma membrane H(+)-ATPase. *Cell Stress Chaperones* **2**: 12-24.

Piper, P.W., Talreja, K., Panaretou, B., Moradas-Ferreira, P., Byrne, K., Praekelt, U.M. *et al.* 1994. Induction of major heat-shock proteins of *Saccharomyces cerevisiae*, including plasma membrane Hsp30, by ethanol levels above a critical threshold. *Microbiology* **140**: 3031-3038.

Posas, F., Chambers, J.R., Heyman, J.A., Hoeffler, J.P., de Nadal, E. & Arino, J. 2000. The Transcriptional Response of Yeast to Saline Stress. *J. Biol. Chem.* **275**: 17249-17255.

Quinteroa, F.J., Blatt, M.R. & Pardo, J.M. 2000. Functional conservation between yeast and plant endosomal Na+/H+ antiporters. *FEBS Lett.* **471**: 224-228.

Quintero, F.J., Ohta, M., Shi, H., Zhu, J-K. & Pardo, J.M. 2002. Reconstitution in yeast of the *Arabidopsis* SOS signaling pathway for Na^+ homeostasis. *Proc. Natl. Acad. Sci. USA* **99**: 9061-9066.

Rancati, G., Pavelka, N., Fleharty, B., Noll, A., Trimble, R., Walton, K. *et al.* 2008. Aneuploidy underlies rapid adaptive evolution of yeast cells deprived of a conserved cytokinesis motor. *Cell* **135**: 879-893.

Reiser, V., Salah, S.M. & Ammerer, G. 2000. Polarized localization of yeast Pbs2 depends on osmostress, the membrane protein Sho1 and Cdc42. *Nat. Cell. Biol.* **2**: 620-627.

Rep, M., Reiser, V., Gartner, U., Thevelein, J.M., Hohmann, S., Ammerer, G. *et al.* 1999. Osmotic Stress-Induced Gene Expression in *Saccharomyces cerevisiae* Requires Msn1p and the Novel Nuclear Factor Hot1p. *Mol. Cell. Biol.* **19**: 5474-5485.

Rep, M., Krantz, M., Thevelein, J.M. & Hohmann, S. 2000. The transcriptional response of *Saccharomyces cerevisiae* to osmotic shock. *J. Biol. Chem.* **275**: 8290-8300.

Ringrose, L. & Paro, R. 2004. Epigenetic regulation of cellular memory by the Polycomb and Trithorax group proteins. *Annu. Rev. Genet.* **38**:413-443.

1144 Rios, G., Ferrando, A. & Serrano, R. 1997. Mechanisms of salt tolerance conferred by
1145 overexpression of the HAL1 gene in *Saccharomyces cerevisiae*. *Yeast* **13**: 515-528.
1146
1147 Rudolph, B., Gebendorfer, K.M., Buchner, J. & Winter, J. 2010. Evolution of *Escherichia*
1148 *coli* for Growth at High Temperatures. *J. Biol. Chem.* **285**: 19029-19034.
1149
1150 Saito, H. & Tatebayashi, K. 2004. Regulation of the Osmoregulatory HOG MAPK
1151 Cascade in Yeast. *J. Biochem.* **136**: 267-272.
1152
1153 Saleh, B., Allario, T., Dambier, D., Ollitrault, P. & Morillon, R. 2008. Tetraploid citrus
1154 rootstocks are more tolerant to salt stress than diploid. *C. R. Biologies*, **331**: 703-710.
1155
1156 Samani, P. & Bell, G. 2010. Adaptation of experimental yeast populations to stressful
1157 conditions in relation to population size. *J. Evol. Biol.* **23**: 791-796.
1158
1159 Sambrook, J. & Russell, D.W. 2001. *Molecular Cloning: A laboratory Manual,* 3rd edn.
1160 Cold Spring Harbor Laboratory Press, New York.
1161
1162 Sanchez, I., Hughes, R.T., Mayer, B.J., Yee, K., Woodgett, J.R. & Avruch, J. *et al.* 1994.
1163 Role of SAPK/ERK kinase-1 in the stress-activated pathway regulating transcription
1164 factor c-Jun. *Nature* **372**: 794-798.
1165
1166 Sanders, D. 2000. Plant biology: The salty tale of *Arabidopsis*. *Curr. Biol.* **10**: R486-
1167 R488
1168
1169 Schmitt, A.P. & McEntee, K. 1996. Msn2p, a zinc finger DNA-binding protein, is the
1170 transcriptional activator of the multistress response in *Saccharomyces cerevisiae. Proc.*
1171 *Natl. Acad. Sci. USA* **93**: 5777-5782.
1172
1173 Schuller, C., Brewster, J.L., Alexander, M.R., Gustin, M.C. & Ruis, H. 1994. The HOG
1174 pathway controls osmotic regulation of transcription via the stress response element
1175 (STRE) of the *Saccharomyces cerevisiae* CTT1 gene. *EMBO J.* **13:** 4382-4389.
1176
1177 Serrano, R. 1996. Salt tolerance in plants and microorganisms: toxicity targets and
1178 defense responses. *Int. Rev. Cytol.* **165**: 1-52.
1179
1180 Serrano, R., Mulet, J.M., Rios, G., Marquez, J.A., de Larrinoa, I.F., Leube, M.P. *et al.*
1181 1999. A glimpse of the mechanisms of ion homeostasis during salt stress. *J. Exp. Bot.*
1182 **50**: 1023-1036.
1183
1184 Stanek, M.T., Cooper, T.F. & Lenski, R.E. 2009. Identification and dynamics of a
1185 beneficial mutation in a long-term evolution experiment with *Escherichia coli*. *BMC Evol.*
1186 *Biol.* **9**:302.
1187

1188 Stebbins, G. L. 1950. Variation and evolution in plants. *Columbia University Press*, New
1189 York.
1190
1191 Tal, M., & Gardi, I. 1976. Physiology of polyploid plants: water balance in autotetraploid
1192 and diploid tomato under low and high salinity. *Physiol. Plantarum* **38**: 257-261.
1193
1194 Teige, M., Scheikl, E., Eulgem, T., Doczi, R., Ichimura, K., Shinozaki, K. *et al.* 2004. The
1195 MKK2 Pathway Mediates Cold and Salt Stress Signaling in *Arabidopsis*. *Mol. Cell* 15:
1196 141-152.
1197
1198 Tsuda, L., Inoue, Y.H., Yoo, M.A., Mizuno, M., Hata, M., Lim, Y.M. et al. 1993. A protein
1199 kinase similar to MAP kinase activator acts downstream of the raf kinase in Drosophila.
1200 Cell 72: 407-414.
1201
1202 Tsujimoto, Y., Izawa, S. & Inoue, Y. 2000. Cooperative Regulation of DOG2, Encoding
1203 2-Deoxyglucose-6-Phosphate Phosphatase, by Snf1 Kinase and the High-Osmolarity
1204 Glycerol–Mitogen-Activated Protein Kinase Cascade in Stress Responses of
1205 *Saccharomyces cerevisiae. J. Bacteriol.* **182**: 5121–5126.
1206
1207 Trainotti, N. & Stambuk, B. U. 2001. NaCl stress inhibits maltose fermentation by
1208 Saccharomyces cerevisiae. *Biotech. Lett.* 23: 1703-1707.
1209
1210 Turner, B.M. 2002. Cellular Memory and the Histone Code. *Cell* **111**: 285-291.
1211
1212 Velicer, G.J., Raddatz, G., Keller, H., Deiss, S., Lanz, C., Dinkelacker, I. *et al.* 2006.
1213 Comprehensive mutation identification in an evolved bacterial cooperator and its
1214 cheating ancestor. *Proc. Natl. Acad. Sci. USA* **103**: 8107-8112.
1215
1216 Vijaranakul, U., Nadakavukaren, M.J., de Jonge, B.L., Wilkinson, B.J. & Jayaswal, R.K.
1217 1995. Increased Cell Size and Shortened Peptidoglycan Interpeptide Bridge of NaCl-
1218 Stressed *Staphylococcus aureus* and Their Reversal by Glycine Betaine. *J. Bacteriol.*
1219 **177**: 5116-5121.
1220
1221 Visscher, P.M. 2008. Sizing up human height variation. *Nat. Genet.* **40**: 489-490.
1222
1223 Visscher, P.M. & Montgomery, G.W. 2009. Genome-wide Association Studies and
1224 Human Disease: From Trickle to Flood. *J. Am. Med. Assoc.* **302**: 2028-2029.
1225
1226 Warringer, J., Ericson, E., Fernandez, L., Nerman, O. & Blomberg, A. 2003. High-
1227 resolution yeast phenomics resolves different physiological features in the saline
1228 response. *Proc. Natl. Acad. Sci. USA* **100: 15724-15729.**
1229
1230 Waskiewicz, A.J. & Cooper, J.A. 1995. Mitogen and stress response pathways: MAP
1231 kinase cascades and phosphatase regulation in mammals and yeast. *Curr. Opin. Cell*

Biol. **7**:798-805.

Wittkopp, P.J., Haerum, B.K. & Clark, A.G. 2004. Evolutionary changes in cis and trans gene regulation. *Nature* **430**: 85-88.

Wu, C-Y., Rolfe, P.A., Gifford, D.K. & Fink, G.R. 2010. Control of Transcription by Cell Size. *PLoS Biol* **8**: e1000523.

Wu, X., Hart, H., Cheng, C., Roach, P.J. & Tatchell, K. 2001. Characterization of Gac1p, a regulatory subunit of protein phosphatase type I involved in glycogen accumulation in *Saccharomyces cerevisiae*. *Mol. Genet. Genomics* **265**: 622-635.

Yale, J. & Bohnert, H.J. 2001. Transcript Expression in *Saccharomyces cerevisiae* at High Salinity. *J. Biol. Chem.* **276**: 15996-16007.

Yan, M., Dai, T., Deak, J.C., Kyriakis, J.M., Zon, L.I., Woodgett, J.R. *et al.* 1994. Activation of stress-activated protein kinase by MEKKI phosphorylation of its activator SEKI. *Nature* **372**: 798-800.

Yang, S.X., Zhao, Y.X., Zhang, Q., He, Y.K., Zhang, H. & Luo, D. 2001. HAL1 mediate salt adaptation in *Arabidopsis thaliana*. *Cell Res.* **11**: 142-148.

Yang, J., Benyamin, B., McEvoy, B.P., Gordon, S., Henders, A.K., Nyholt, D.R. *et al.* 2010. Common SNPs explain a large proportion of the heritability for human height. *Nat. Genet.* **42**: 565-569.

Yashar, B.M., Kelley, C., Yee, K., Errede, B. & Zon, L.I. 1993. Novel Members of the Mitogen-Activated Protein Kinase Activator Family in *Xenopus laevis*. *Mol. Cell. Biol.* **13**: 5738-5748.

Zacharioudakis, I., Gligoris, T. & Tzamarias, D. 2007. A Yeast Catabolic Enzyme Controls Transcriptional Memory. *Curr. Biol.* **17**: 2041–2046.

Zamanillo, D., Hunt, T. & Nebreda, A.R. 1994. A novel kinase cascade triggered by chemical stress and heat shock which stimulates MAP kinase-activated protein kinase-2 and phosphorylation of the small heat shock proteins. *Cell* **78**: 1027-1037.

Zeggini, E., Scott, L.J., Saxena, R., Voight, B.F., Marchini, J.L., Hu, T. *et al.* 2008. Meta-analysis of genome-wide association data and large-scale replication identifies additional susceptibility loci for type 2 diabetes. *Nat. Genet.* **40**: 638-645.

Zeyl, C., Vanderford, T. & Carter, M. 2003. An Evolutionary Advantage of Haploidy in Large Yeast Populations. *Science* **299**: 555-558.

Zhang, Y., Fatima, N. & Dufau, M.L. 2005. Coordinated Changes in DNA Methylation and Histone Modifications Regulate Silencing/Derepression of Luteinizing Hormone Receptor Gene Transcription. *Mol. Cell. Biol.* **25**: 7929-7939.

Zheng, D.Q., Wu, X.C., Tao, X.L., Wang, P.M., Li, P., Chi, X.Q., Li, Y.D., Yan, Q.F. & Zhao, Y.H. 2011. Screening and construction of Saccharomyces cerevisiae strains with improved multi-tolerance and bioethanol fermentation performance. *Bioresour. Technol.* 102:3020-3027.

Zhu, J-K. 2001. Plant salt tolerance. *Trends Plant Sci.* **6**: 66-71.

Zhu, J-K. 2002. Salt and Drought Stress Signal Transduction in Plants. *Annu. Rev. Plant. Biol.* **53**: 247-273.

Figures

Figure 1 - (a-c) Viability of the ancestral strain and the evolved lines in medium containing 0.5M NaCl. Cells from the ancestral strain and the evolved lines from overnight cultures were transferred into YPG medium. Cell samples from these cultures were withdrawn after 16 hours (during exponential growth phase) as well as after 24 hours (stationary phase), and then diluted and plated. The plates were incubated at 30ºC for five days and the number of colonies were counted. The relative viability of both the ancestral strain and the evolved lines were estimated from the ratio of colonies formed on agar plates with YPGN medium to that of plates with YPG medium. Relative viabilities were measured for (a) the ancestral strain, (b) lines evolved in YPGN medium (S lines), and (c) lines evolved in YPG medium (C lines). From the figures, it is clear that NaCl does not affect the viability of the ancestral strain. The viabilities of the evolved lines S1, S2 and S3 do not increase during the course of adaptation.

Figure 2 - Fitness Assay of the evolved yeast lines. Fitness assays were performed by inoculating equal numbers of cells from an evolved line and from the GFP-tagged reference yeast strain in the same vessel. The cells were grown for 24hrs, and the percentage of non-GFP-tagged cells was counted using FACS. The ratio of the cell numbers of the evolved lines to that of the ancestral strain gives the relative fitness of the evolved lines. (a) Relative fitness of the three replicate yeast lines evolved in medium with salt (S1,S2 and S3) in YPGN medium, and the three replicate yeast lines evolved without NaCl (C1,C2,C3) measured in YPGN medium. (b) Relative fitness of the lines S1, S2 and S3 in medium without NaCl. The lines evolved in salt show an increase in growth rate by 8% to 12% in NaCl medium compared to the ancestral strain. These lines also show a growth rate increase in medium without NaCl. However, this increase in growth rate is consistently lower than the increase in NaCl medium. In addition, the lines evolved in medium without NaCl show lower fitness in NaCl medium. Taken together, it can be concluded that part of the adaptation in the salt evolved lines is specific to NaCl.

Figure 3 - The genes showing similar (t-test, p-value<0.05, False Discovery Rate<10%) induction or repression by NaCl in the ancestral strain and in the evolved S lines (a) Volcano plot, showing the p-values on the Y axis, and the fold expression change for these genes on the X axis. The labeled genes include genes associated with the stress response (e.g., *GPD1, SIP18*), genes with high level of expression change (e.g., *FMP48, NOG1*), and genes in both categories (e.g., *GRE1*). (b) Scatter plot for the fold-change of the common genes in the ancestral strain (X-axis) and in the evolved S lines (Y-axis). Although most of the common genes change expression to a similar extent in the ancestral strain and the S lines, a small number of genes change expression to a different extent.

Figure 4 - Schematic diagram for the type of expression change that can occur as a result of evolutionary adaptation in the evolved S lines compared to the ancestral strain. Each bar chart reflects the expression level of a hypothetical gene in the ancestral and evolved strain, in the absence and presence of salt. The scenarios that are depicted are as follows (a) No basal change, increased regulation (b) No basal change, increased repression (c) Basal increase in expression but no change in regulation (d) Basal decrease in expression but no change in regulation.

Figure 5 - Genes with differential expression in the S lines compared to the ancestral strain (|Z-score|≥1.5). The plots show the level of expression change (vertical axis) and the Z-score (horizontal axis) for genes with (a) changed regulation, and (b) changed basal expression in the evolved lines. One should note that the basal change in expression is represented in terms of fold change, where the expression change is calculated as the ratio of the expressions in the evolved lines and the ancestral strain. The calculation of regulated expression change involves an additional subtraction of basal expression change and thus, can not be represented as fold change.

Figure 6 - The five genes with the highest Z-score in each of four categories of expression change in the evolved lines. Categories are (a) change in the level of induction, (b) change in the level of repression, (c) increase in basal expression, and (d) decrease in basal expression. The columns show, from left to right, the systematic gene name, the extent of expression change, the Z-score, the gene's common name, the type of expression change (in (a) and b) only), and a reference, if the gene had responded physiologically to hyperosmotic stress or saline stress in previous studies.

Figure 7 - Different functional gene classes (Güldener *et al.*, 2005) are enriched or impoverished for genes that change expression in the salt evolved yeast lines (S lines), compared to the ancestral strain. The vertical axis shows the functional classes of genes we consider, and the horizontal axis shows the fraction of genes in each category listed on the bottom of the figure. Asterisks ('*') indicate classes where the number of genes differs significantly from that expected by chance alone, as indicated by an exact binomial test.

Figure 8 - Fitness analysis (using FACS) of the ancestral strain into which the mutant *MOT2* gene of line S2 was introduced, and the corresponding wild-type *MOT2* gene as a control. (a) Fitness in medium with NaCl (b) Fitness in medium without NaCl. The data show that the mutant *MOT2* gene increases the fitness in salt medium as well as in medium without salt, and to similar extents.

Figure 9 - Pulsed field gel electrophoresis (PFGE) analysis of the three replicate salt evolved lines S1,S2 & S3 and the ancestral strain. Two clones from each of the lines S1,S2 and S3 were subjected to PFGE analysis along with the ancestral strain. (a) The PFGE for the yeast chromosomes from the evolved lines S1, S2 and S3 and the ancestral strain. The chromosomes are shown on the left side. M represents the yeast chromosome PFG marker (NEB Catalog# N0345S). Only line S3 shows a new band (red arrow) of size approximately 500kb. (b) PFGE analysis of the *Not*I digested chromosomes from the lines S1,S2,S3 and the ancestral strain. M represents the Mid-range PFG marker I from NEB (Catalog# N3551S), whose fragments range between 15 kb and 291 kb. Again, only line S3 shows a new band (red arrow) in the gel, which has a size of approximately 180kb.

Figure 10 - DNA content per yeast cell of the ancestral strain and the evolved lines.
The ancestral strain and population samples of the evolved lines were grown for 24 hours at 30^{o}C. For each of the cultures, the cell density was estimated. Genomic DNA was isolated from a defined number of cells and DNA quantification was carried out with a NanoDrop ND-1000 spectrophotometer. The DNA content per cell was then calculated for each line. The figure suggests that all the evolved lines have become massively aneuploid.

Figure 11 - Cell size comparison between ancestral strain and one evolved line. (a) Histogram of cell diameters of the ancestral strain and the S2 line, showing that the salt evolved cells are bigger than the ancestral cells. (b) Microphotograph of cell samples from the ancestral strain and the salt evolved lines.

Figure 1:

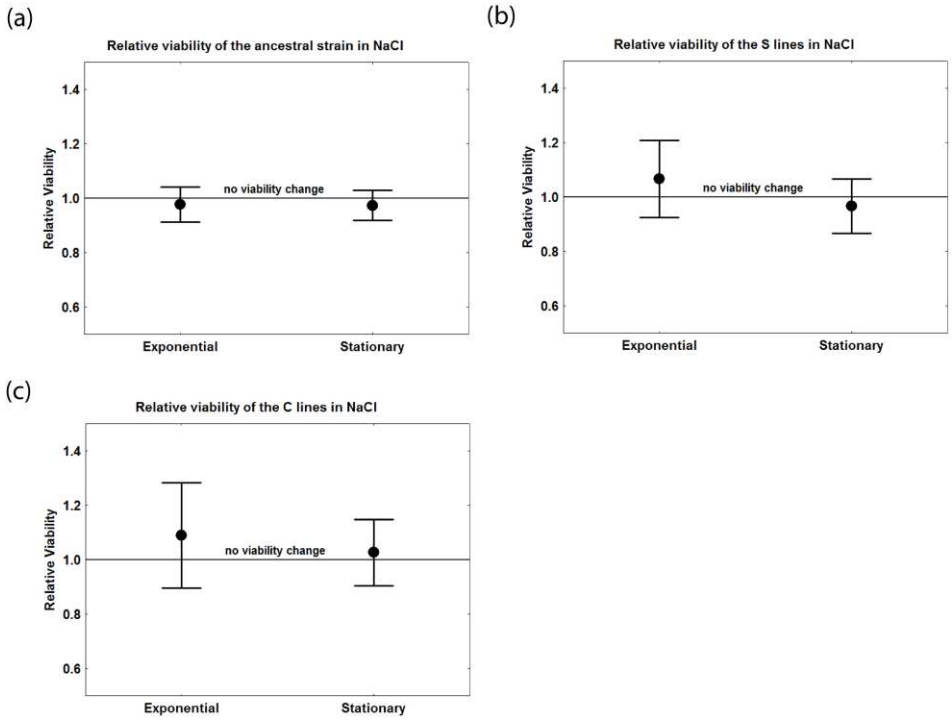

(a)
Relative viability of the ancestral strain in NaCl
Relative Viability
1.4
1.2
1.0
0.8
0.6
no viability change
Exponential
Stationary
(b)
Relative viability of the S lines in NaCl
Relative Viability
1.4
1.2
1.0
0.8
0.6
no viability change
Exponential
Stationary
(c)
Relative viability of the C lines in NaCl
Relative Viability
1.4
1.2
1.0
0.8
0.6
no viability change
Exponential
Stationary

Figure 2:

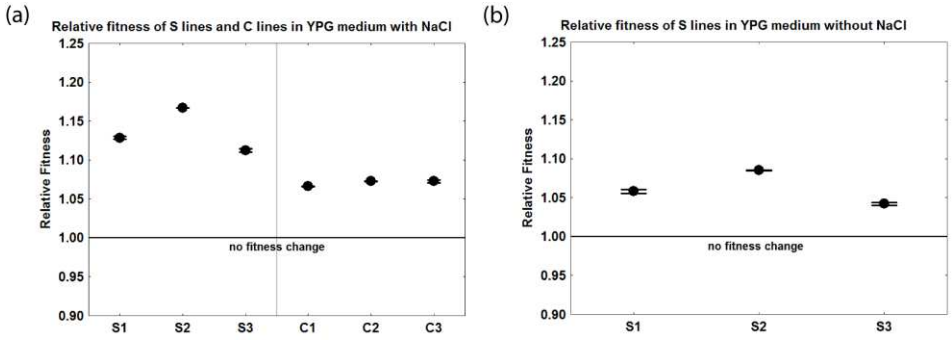
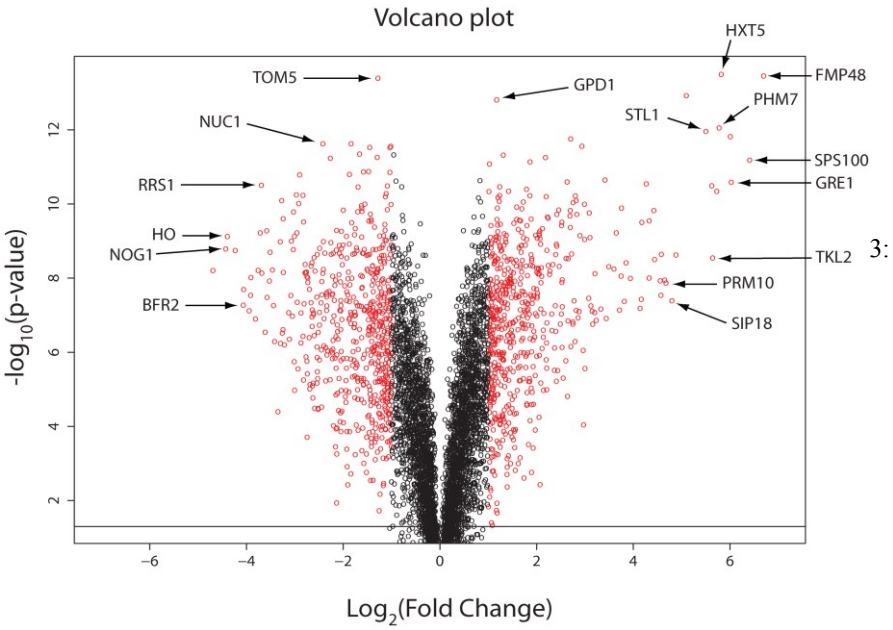


Figure 3:

(a)



(b)

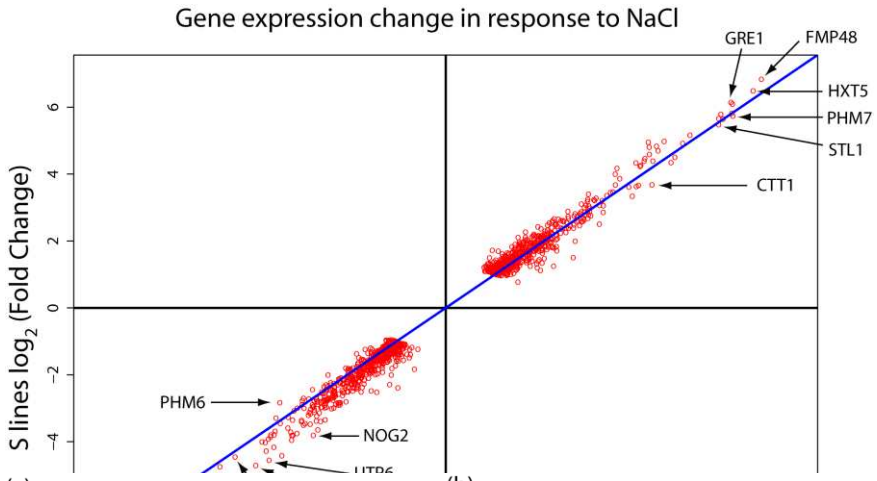
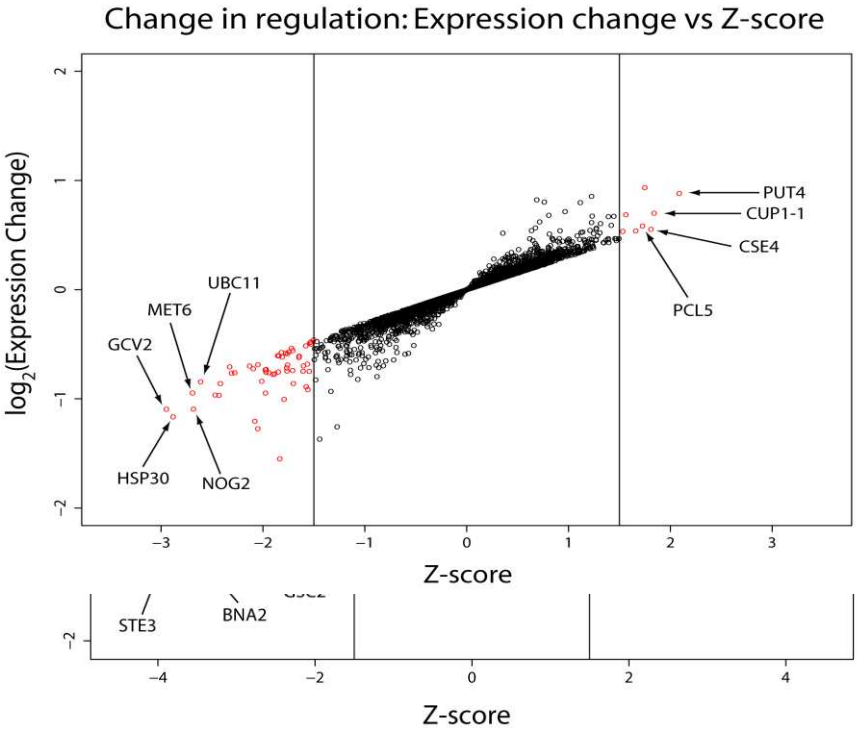

Gene expression change in response to NaCl
S lines log2 (Fold Change)
GRE1
FMP48
HXT5
PHM7
STL1
CTT1
PHM6
NOG2
(a)
(b)
No basal change, increased induction
No basal change, increased repression
Ancestral
Evolved
Expression Level
-NaCl
+NaCl
(c)
(d)
Basal increase, no change in regulation
Basal decrease, no change in regulation

Figure 4:

Figure 5:

(a)


Change in regulation: Expression change vs Z-score
log2(Expression Change)
Z-score
PUT4
CUP1-1
CSE4
PCL5
UBC11
MET6
GCV2
HSP30
NOG2
STE3
BNA2
Z-score

(b)

Figure 6:

(a) Top five genes with change in induction level

Gene	Log2 (Expression Change)	Z-score	Common Name	Type of change	Previous studies
YOR348C	0.8821	2.088	PUT4	Increased induction	Posas et al., 2000; Rep et al., 2000; Yale & Bonhert, 2001
YHR053C	0.7017	1.8401	CUP1-1	Increased induction	Yale & Bonhert, 2001
YKL049C	0.554	1.8101	CSE4	New induction	Yale & Bonhert, 2001
YAR010C	0.9355	1.7493	---	New induction	Yale & Bonhert, 2001
YHR071W	0.5834	1.726	---	Increased induction	Yale & Bonhert, 2001

(b) Top five genes with change in repression level

Gene	Log2 (Expression Change)	Z-score	Common Name	Type of change	Previous studies
YMR189W	-1.0952	-2.9498	GCV2	New repression	Yale & Bonhert, 2001
YER091C	-0.9458	-2.6921	MET6	Increased repression	Rep et al., 2000
YNR053C	-1.0932	-2.6838	NOG2	Increased repression	---
YOR339C	-0.8424	-2.613	UBC11	Increased repression	---
YHR066W	-0.9644	-2.4702	SSF1	Increased repression	---

(c) Top five genes with increase in basal expression

Gene	Log2 (Fold Change)	Z-score	Common Name	Previous studies
YNL034W	1.3572	4.2657	---	---
YOR384W	1.2084	3.8216	FRE5	---
YOR049C	1.4081	3.5885	RSB1	---
YGL192W	1.5698	3.3941	IME4	---
YOR382W	1.8809	3.3935	FIT2	Yale & Bonhert, 2001

(d) Top five genes with decrease in basal expression

Gene	Log2 (Fold Change)	Z-score	Common Name	Previous studies
YKL178C	-1.2774	-3.9263	STE3	---
YML058W-A	-1.2118	-3.7933	HUG1	---
YJR078W	-1.2138	-3.5631	BNA2	---
YER011W	-1.0741	-3.361	TIR1	---
YDR281C	-1.0859	-2.789	PHM6	---

Figure 7:

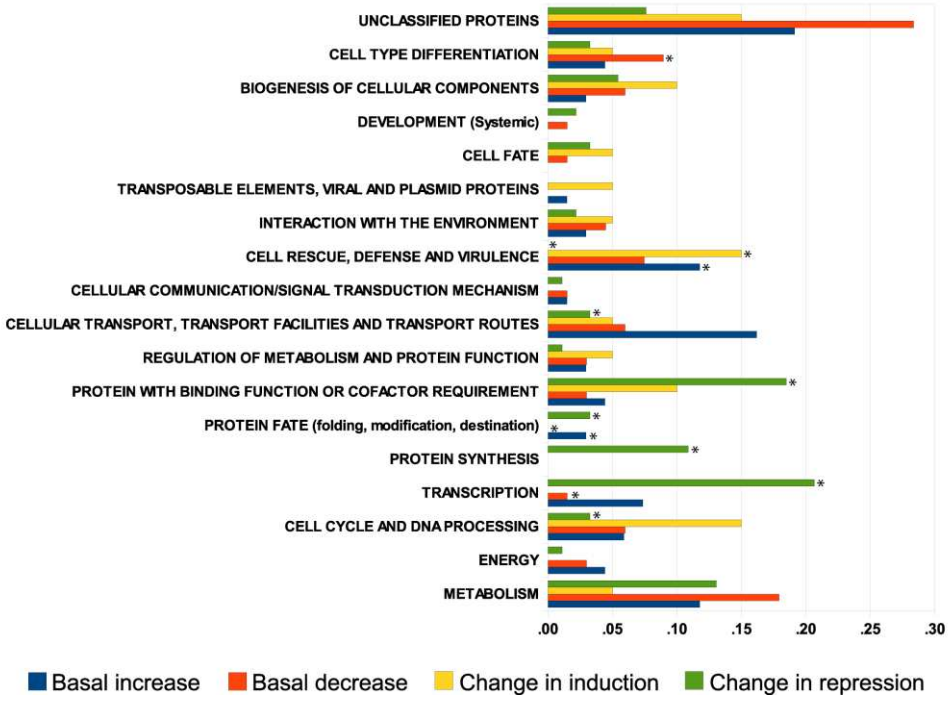

UNCLASSIFIED PROTEINS
CELL TYPE DIFFERENTIATION
BIOGENESIS OF CELLULAR COMPONENTS
DEVELOPMENT (Systemic)
CELL FATE
TRANSPOSABLE ELEMENTS, VIRAL AND PLASMID PROTEINS
INTERACTION WITH THE ENVIRONMENT
CELL RESCUE, DEFENSE AND VIRULENCE
CELLULAR COMMUNICATION/SIGNAL TRANSDUCTION MECHANISM
CELLULAR TRANSPORT, TRANSPORT FACILITIES AND TRANSPORT ROUTES
REGULATION OF METABOLISM AND PROTEIN FUNCTION
PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT
PROTEIN FATE (folding, modification, destination)
PROTEIN SYNTHESIS
TRANSCRIPTION
CELL CYCLE AND DNA PROCESSING
ENERGY
METABOLISM
.00
.05
.10
.15
.20
.25
.30
Basal increase
Basal decrease
Change in induction
Change in repression

Figure 8:

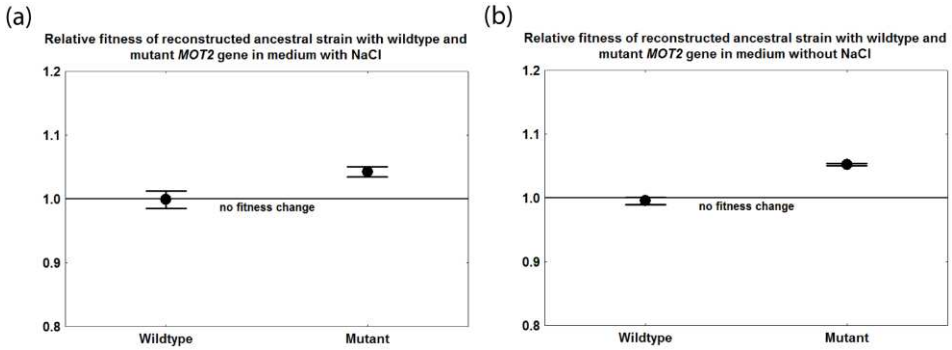

(a)
Relative fitness of reconstructed ancestral strain with wildtype and mutant MOT2 gene in medium with NaCl
1.2
1.1
1.0
0.9
0.8
no fitness change
Wildtype
Mutant
(b)
Relative fitness of reconstructed ancestral strain with wildtype and mutant MOT2 gene in medium without NaCl
1.2
1.1
1.0
0.9
0.8
no fitness change
Wildtype
Mutant

Figure 9:

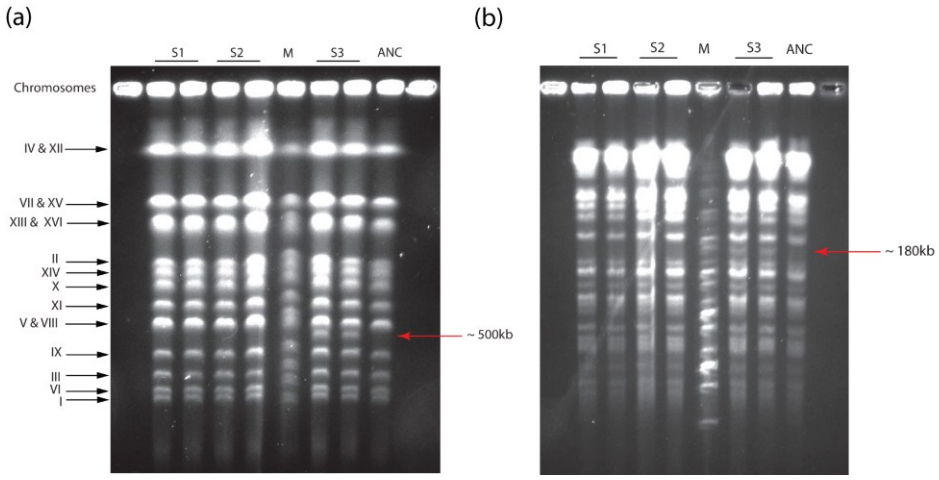

(a)
(b)
S1
S2
M
S3
ANC
Chromosomes
IV & XII
VII & XV
XIII & XVI
II
XIV
X
XI
V & VIII
IX
III
VI
I
~ 500kb
~ 180kb

Figure 10:

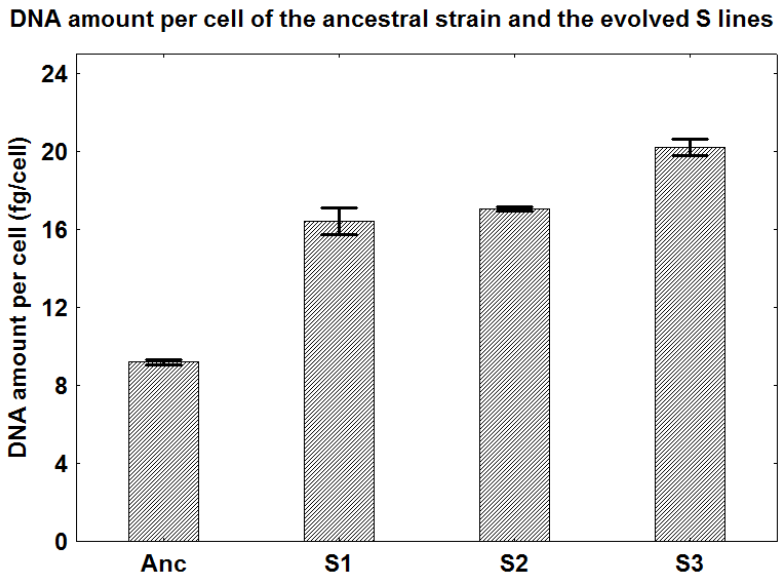
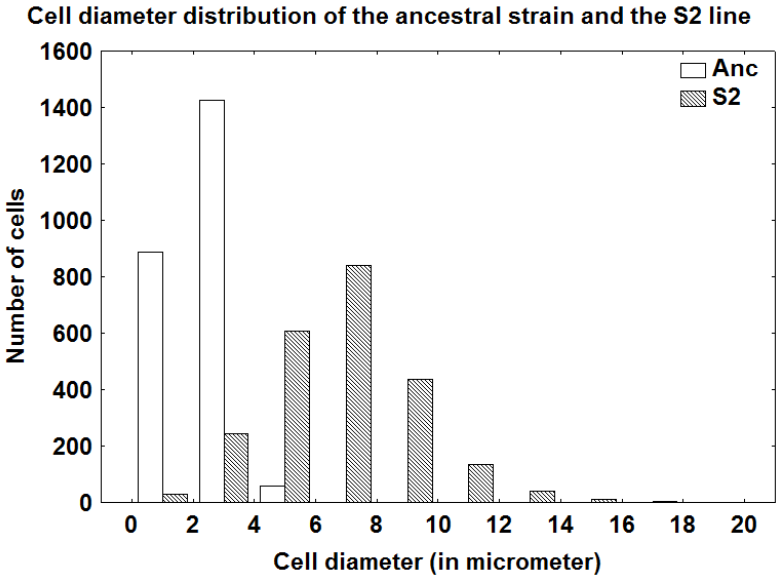


Figure 11:

(a)



(b)